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PRINCIPAL INVESTIGATOR: Khandan Keyomarsi, Ph.D.

CONTRACTING ORGANIZATION: Health Research, Inc.
Rensselaer, New York 12144-3456

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13. ABSTRACT *(Maximum 200*
We have examined tumor specimens from 400 breast cancer patients, comparing the changes of cyclin E expression with seven other established tumor markers as well as patient outcome. Altered expression of cyclin E was observed in 90% of all breast cancers with poor prognosis where patients either died of breast cancer or were still with cancer at the last contact date. Similarly in 85% of all breast cancer patients where cyclin E was either not altered, or its alteration was minimal, patients had a favorable prognosis. We also examined the expression of cyclin D and PCNA in the same group of patients and found while cyclin D was overexpressed in40% of all cases examined, such overexpression was not linked to poor prognosis or to cyclin E overexpression. Lastly, overexpression of PCNA, indicative of highly proliferative cancer, was not sufficient to account for such high association of cyclin E expression to poor prognosis. These analysis revealed that cyclin E protein is the most consistent marker for determining the prognosis of early-stage node-negative ductal carcinoma, providing very strong evidence for the use of cyclin E as a novel prognostic marker for breast cancer. The alteration of cyclin E in breast cancer has been further characterized and reveals that while cyclin E is cell cycle regulated in normal cells, it is present constitutively and in an active complex in synchronized populations of breast cancer cells. Since cyclin E is active throughout the cell cycle in tumor and not normal cells, it functions redundantly and activates substrates in only tumor cells.

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K. Keyserman / for 11/24/97

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5. Introduction:

The overall purpose of this 4 year study is to use the altered expression of cyclin E as a diagnostic/prognostic marker and to investigate the mechanisms and repercussions of this alteration in breast cancer.

Cyclins are prime cell cycle regulators and central to the control of cell proliferation in eukaryotic cells via their association with and activation of cyclin-dependent protein kinases 1-7 (cdks) (reviewed in [1-6]. Cyclins were first identified in marine invertebrates as a result of their dramatic cell cycle expression patterns during meiotic and early mitotic divisions [7-10]. Several classes of cyclins have been described and are currently designated as cyclins A-H, some with multiple members. Cyclins can be distinguished on the basis of conserved sequence motifs, patterns of appearance and apparent functional roles during specific phases and regulatory points of the cell cycle in a variety of species. The cdk partners of several of these cyclins have also been identified: Cyclin A forms a complex with cdc2 (cdk1) and cdk2, and is required both at mitosis and DNA replication [11-14]; cyclin B forms a complex solely with cdc2 and is required for entry into mitosis, (reviewed in [3]; cyclin D1, a cyclin active in the G1 phase of the cell cycle, forms complexes primarily with cdk4 and cdk6, while cyclin E, another G1 type cyclin, forms a complex with only cdk2 [3, 4, 15-19]. Lastly, cyclin H has been shown to form a complex with cdk7 and, together, they comprise the cdk-activating kinase (CAK) protein complex which activates the nascent cyclin/cdk complex via phosphorylation [20, 21]. Cyclin binding to a cdk enables the kinase to become active, initiating a complex kinase cascade that directs the cell into DNA synthesis and/or mitosis, reviewed in [15, 22].

An additional layer of cell cycle regulation has emerged with the discoveries of low molecular weight cdk inhibitors (CKIs) which represent a novel mode of negative regulation [23-25]. The

first class of these inhibitors, p21, was simultaneously characterized in several laboratories as the major p53 inducible gene (WAF1) [26-28], as a CDK inhibitor protein (CIP1, p21, and $p20^{CAP1}$) [29-31], as a protein highly expressed in senescent fibroblasts (sdi) [32], and as a melanoma differentiation associated gene (mda6) [33]. In normal fibroblasts, this protein has been shown to be associated with and inhibit various cyclin-cdk complexes, including cdk2 associated with cyclins A and E, cdk4 associated with D-type cyclins [29, 30, 34-36], and is also found weakly associated cdc2-cyclin B [34]. This protein which represents one of the major p53 inducible genes, is also induced during differentiation. It most likely acts as a general purpose brake used during terminal differentiation and p53 directed DNA damage control [37]. The second protein in this family, $p27^{KIP1}$, is both structurally and functionally similar to p21. $p27^{KIP1}$ was identified simultaneously as a protein associated with inactive cyclin E-cdk2 complexes in TGFß treated and contact inhibited cells [38, 39] and as a protein that interacts with cyclin D1-cdk4 complexes [40]. TGFß arrests certain cell types in G1 and p27 is thought to be a cellular mediator for this anti-proliferative signal [41]. Hence, p21 and p27 may function similarly to inhibit cdk activity and proliferation in response to different environmental stimuli.

A second, structurally and functionally distinct family of CKIs is comprised of p16, p15 and their homologous [34, 42-45]. Structural features of these Ink4 (for inhibitor of cdk4) proteins include 4 ankyrin like repeats which are postulated to be involved in mediating protein-protein interactions [43, 46]. Curiously these CKIs share significant homology to the Notch proteins involved in the differentiation and fate determination of cells during embryogenesis [42]. Inhibitors of this family bind cdk monomers (cdk4 or cdk6) rather than cyclin-cdk complexes [34, 46]. It is believed that binding of ink4 proteins to cdks prevents and/or disrupts cyclin-cdk complex formation thereby negating cdk activity. p16 and p15 proteins, encoded on human chromosome 9, have been the subjects of intense study as this genomic region is frequently mutated in a variety of tumor cell lines and fewer tumor tissue specimens [34, 46]. As their alternate names imply (MTS1 and

MTS2 for multiple tumor suppressor) p16 and p15 are postulated to function as growth inhibitory tumor suppressor molecules.

The connection between cyclins CKIs and cancer has been substantiated with the D type cyclins [6, 9, 22, 47]. Cyclin D1 was identified simultaneously by several laboratories using independent systems: It was identified in mouse macrophages due to its induction by colony stimulating factor 1 during G1 [48]. It was also identified in complementation studies using yeast strains deficient in G1 cyclins [15, 49]; as the product of the bcl-1 oncogene [50], and as the PRAD1 proto-oncogene in some parathyroid tumors where its locus is overexpressed as a result of a chromosomal rearrangement that translocates it to the enhancer of the parathyroid hormone gene [48, 51-53]. In centrocytic B cell lymphomas cyclin D1 (PRAD1)/BCL1 is targeted by chromosomal translocations at the BCL1 breakpoint, t(11;14)(q13;q32) [54, 55]. Furthermore, the cyclin D1 locus undergoes gene amplification in mouse skin carcinogenesis, as well as in breast, esophageal, colorectal and squamous cell carcinomas [56-62]. Several groups have examined the ability of cyclin D1 to transform cells directly in culture with mixed results [9, 51, 61, 63-67]. However, the overexpression of cyclin D1 was recently observed in mammary cells of transgenic mice and results in abnormal proliferation of these cells and the development of mammary adenocarcinomas [68]. This observation strengthens the hypothesis that the inappropriate expression of a G1 type cyclin may lead to loss of growth control.

Cyclins D2 and A have also been implicated in oncogenesis. The cyclin D2 gene appears to be the integration site of a murine leukemia provirus in mouse T cell leukemias, resulting in its overexpression [69]. Cyclin A was found to be the site of integration of a fragment of the hepatitis B virus genome in a hepatocellular carcinoma [70]. Cyclin A is also associated with the adenovirus transforming protein E1A in adenovirus transformed cells [71, 72]

The linkage between oncogenesis and the cell cycle was recently reinforced by correlating the deranged expression of cyclins to the loss of growth control in breast cancer [58, 73]. Using proliferating normal versus human tumor breast cell lines in culture as a model system, several changes were seen in all or most of these lines. These include increased cyclin mRNA stability, resulting in overexpression of mitotic cyclins and cdc2 RNAs and proteins in 9/10 tumor lines, leading to the deranged order of appearance of mitotic cyclins prior to G1 cyclins in synchronized tumor cells. The most striking abnormality in cyclin expression was that of cyclin E. Cyclin E protein not only was overexpressed in 10/10 breast tumor cell lines but it was also present in lower molecular weight isoforms than that found in normal cells [73]. The relevance of cyclin derangement to *in vivo* conditions, was directly examined by measuring the expression of cyclin E protein in tumor samples versus normal adjacent tissue obtained from patients with various malignancies [74]. These analyses revealed that breast cancers and other solid tumors, as well as malignant lymphocytes from patients with lymphatic leukemia, show severe quantitative and qualitative alteration in cyclin E protein expression independent of the S-phase fraction of the samples. In addition, the alteration of cyclin E becomes more severe with breast tumor stage and grade and is more consistent than cell proliferation or other tumor markers such as PCNA or c-erb B2. These observations strongly suggested the use of cyclin E as a new prognostic marker. These findings were corroborated by immunocytochemical detection of cyclin E which detects tumor proliferation and deregulated cyclin expression. The mechanism of the cyclin E alteration is in part a result of its deregulation in breast cancer. The alteration of cyclin E in breast cancer have been recently further characterized and reveal that while cyclin E is cell cycle regulated in normal cells it is present constituitively and in an active cdk2 complex in synchronized populations of breast cancer cells. Two novel truncated variant forms of cyclin E mRNA as detected by RT-PCR were also identified which are ubiquitously detected in normal and tumor cells and tissues. These variant forms of cyclin E can give rise to an active cyclin/cdk2 complex *in vitro*, but they do not seem to be translated in normal cells.

During the first three years of this application we have used cyclin E antibody as a prognostic marker for breast cancer by analyzing breast tumor tissue specimens for the alterations in cyclin E protein. During the first two years we collected 550 tumor tissue samples from breast cancer patients diagnosed with different stages of breast cancer ranging from pre-malignant to highly invasive. We extracted RNA, DNA and protein from most of these samples. Due to limited sample size received for each patient (i.e. 0.1-0.2 g of tissue), protein was initially extracted from all samples and if there was tumor sample left over, DNA and RNA were also extracted. The protein extracts from all 500 samples were then subjected to Western blot analysis and the expression of cyclin E was compared and correlated with other known prognostic markers examined in the same samples. The prognostic markers include, cyclin D1, erbB-2, as well as PCNA to determine the proliferative activity of these samples. We also obtained information on the estrogen and progesterone receptor status of each sample as well as ploidy and proliferation rate as measured by Ki-67. In the second year of this study we analyzed the results obtained in the first year by quantitating the levels of cyclin E in each tumor specimen with that of cyclin D1, erbB2 and PCNA. These analysis were done by performing densitometric scanning on each lane of each gel with each antibody for each patient sample using at least two autoradiographs with different exposures. Such laborious analysis were necessary to accurately determine the level of cyclin E protein in every patient and correlate the alteration of cyclin E protein from each patient to the stage of their disease. During the third year of this application we contacted the 20 hospitals where these samples were obtained, and have been successful in collecting the following information on 385 of these patients: final diagnosis, TNM staging, treatment given, and final outcome (i.e. quality of survival). Having all this information we have performed correlative analysis on these samples and evaluated the role of cyclin E as a prognosticator for breast cancer

During the third year of this application we have also developed a new antibody to cyclin E which can be used for detection of the alteration of cyclin E in immunohistochemical analysis using tissue slides obtained from frozen tissue samples.

In addition to the clinical research outlined above we have also engaged in studies to determine the mode of regulation of cyclin E in normal cells and how this regulation is altered in cancer cells. During the second and third years of the grant we documented that cyclin E is in fact deregulated in breast cancer and such deregulation gives rise to redundancy in function. We show that under conditions where cyclin E is overexpressed it can act redundantly and replace cyclin D as well as cyclin A in breast cancer cell lines. We also document that such redundancy is also seen in tumor tissue specimen [75]. During the third year of the application we have documented that not only are the expression of CKIS including $p21^{CIP1}$ in normal versus tumor cells are different, but the CKIs can be pharmacologically induced in tumor cells and that such overexpression could lead to induction of the estrogen receptor in otherwise estrogen receptor negative breast tumor cells [76].

6: Body (Results/Discussion)

Use of cyclin E as a prognostic marker for breast cancer: During the third year of this grant application we finished the experimental section of the first 2 Specific Aims and continued studies as outlined on Specific Aims 3 and 4. For the first study we collected 550 breast cancer specimens, analyzed cyclin E on 500 of these samples, and were able to obtain clinical and outcome data on 385 of these cases. We were able to analyze the expression of Cyclin E, Cyclin D1, and PCNA on Western blot analysis and correlate their expression to prognosis and outcome of patients. In figure 1 we are presenting the demographic and multivariate analysis of cyclin E in the 385 patients. For each patient we were able to obtain the following clinical information: age, diagnosis, Estrogen and Progesterone receptor status, DNA index, ploidy, proliferation index, clinical stage, treatment received, and outcome of the patients (Fig 1A). We also analyzed each sample by Western blot analysis using cyclin E, two different cyclin D1 antibodies, PCNA and actin (Fig 1A). As described below, we also developed a new antibody for cyclin E which we can use in immunohistochemical analysis and examined cyclin E levels in tissue section of frozen tissues left over from 150 samples (the rest of the samples were used up in Western blot analysis) and compared the results to those obtained from Western blot analysis. The initial demographic

data from the patient populations is also presented in figure 1B. Of the 385 cases examined who were diagnosed between 1990 and 1995, 95 were below the age of 50 and 290 were above the age of 50 and 60-70% of the patients were estrogen receptor positive, while 30-40% were estrogen receptor negative. The TNM stage groupings of the patients revealed that 30% of the patients were diagnosed with Stage I, 49% with Stage II, 14% with Stage 3, and 7.5% with Stage 4 of disease. A representative Western blot analysis of the patients is shown in figure 2 where expression of cyclin E was examined in 12 patients diagnosed with different stages of breast cancer. The first two lanes of the gel are used as controls and the samples are from normal and tumor-derived breast epithelial cells. Every single gel used for Western blot analysis contained the same normal versus tumor control lanes to ensure proper experimental conditions and similar exposures for each Western blot was used for comparative analysis between the blots. For the experiment presented in figure 2, frozen tissue samples obtained from breast cancer patients were minced, homogenized, sonicated, analyzed for total protein (data not shown) and equal amounts of protein was subjected to Western blot analysis using antibodies to Cyclin E, Cyclin D1, PCNA, and actin which was used to monitor of equal loading. It is evident that as the stage of the cancer increases so does the alteration in cyclin E expression. In tumor tissues from stage I disease, overall cyclin E expression was low and the appearance of the lower molecular weight isoforms of cyclin E was minimal. In tumor samples from stage II patients, the expression of cyclin E and the appearance of the lower molecular weight isoforms of cyclin E were detectable and showed a visible increase in intensity compared to stage I tumors. In stage III tumor tissues, cyclin E expression was much more abundant and the levels of the lower molecular weight isoforms also increased substantially. Lastly, in the metastatic stage IV disease we observe severe overexpression of cyclin E and a significant overexpression of the lower molecular weight isoforms of cyclin E. Clearly, the expression of cyclin E increases quantitatively and qualitatively with the stage of the disease. We also examined the expression of cyclin D1 and PCNA in the same tissue samples by stripping and reprobing the blots with the aforementioned antibodies. As evident the expression of cyclin D1, another cyclin active in the G1 phase of the cell cycle and thought to have oncogenic potential, was

not correlated with the stage of the disease. In fact tumor tissues from Stage I patients who had low cyclin E levels but high proliferation index as indicated by PCNA expression also had high cyclin D1 levels. Hence, cyclin D1 expression although increased according to the proliferation index of the tumors, did not increase with the stage of the disease. The expression of PCNA, proliferating cell nuclear antigen which is often used to measure the proliferation index of a cell or tissue, revealed that although overall the rate of proliferation of tissues with high stage tumors were maximal, low stage tumors can also be highly proliferative. Hence, of the markers examined cyclin E was the most consistent indicator for the stage of the disease independent of the proliferation rate of the tissue being examined. We next correlated the overexpression of cyclin E and cyclin D1 to patient outcome and the results are presented in figure 3. Of the 385 cases for which we had all the clinical and research data, 286 patients had low cyclin E expression and 99 of the patients had high cyclin E expression. Of the patients with low cyclin E expression 90% had good prognosis where the patient has no evidence of disease and is alive and well. Of the 99 cases with high cyclin E expression 88% had poor prognosis where the patient either expired of cancer or the cancer is back in the form of metastasis. Hence, cyclin E overexpression is clearly a good and novel prognosticator for predicting outcome of the patients. On the other hand cyclin D1 was not a good prognosticator of patient outcome because even though cyclin D1 was high in 187/385 patients its overexpression did not correlate with patient outcome as 60% of those cases with high cyclin D1 had good outcome while 40% had poor outcome. While these results were very exciting and indicated a prognostic role for cyclin E it was crucial to determine if cyclin E is an independent predictor of poor outcome or just another marker for predicting stage of the disease. For that purpose we correlated the overexpression of cyclin E to the stage of the disease and examined patient outcome for each stage (Fig 4). Cyclin E overexpression leading to patient mortality was observed in every stage of the disease. Of the 99 cases overexpressing cyclin E, 11 cases were diagnosed with stage I and all 10 patients expired of cancer. There were 37 patients diagnosed with stage II disease overexpressing cyclin E and 35 of those patients died of the disease. There were 32 patients overexpressing cyclin E with stage III disease and 25 of them died

of cancer and lastly of the 19 patients diagnosed with stage IV of the disease overexpressing cyclin E 16 expired of metastatic cancer. This figure reveals two observations: First, overexpression of cyclin E is an independent marker from the stage of the disease since patients diagnosed with all 4 stages of the disease whose tumor overexpressed cyclin E had a high chance of poor outcome. Secondly, overexpression of cyclin E could be used as a signal for more aggressive therapy. This conclusion is based on the observation that the only group of patients overexpressing cyclin E who survived were those who were diagnosed with stage III or IV and because of the high stage of their disease received more aggressive therapy. Therefore we speculate that because of the aggressive treatment these cohort of patients overexpressing cyclin E survived the disease. This prediction is further substantiated with the observation that most of the patients in stage I and II of the disease overexpressing cyclin E who had otherwise favorable predictors did not survive the disease due to more conservative therapy. For example the steroid status of most of these stage I and stage II were positive, the patients were lymph node negative, ploidy was near diploid and proliferation was low, yet cyclin E levels were high. Most of these patients were treated with surgery alone or surgery combined with hormonal therapy which was not affective. We suggest that if the expression of cyclin E could have also been used as a predictor, the patient may have been treated more aggressively and the outcome could have been altered. From these results we suggest that cyclin E is a novel prognosticator for breast cancer and could be used in better management of the disease in terms of treatment.

One of the challenges with using cyclin E as a prognostic marker for breast cancer is the method by which it is detected, i.e. Western blot analysis. Although Western blot analysis is a very sensitive technique and can detect not only the overexpression of the cyclin E but the appearance of its lower molecular weight isoforms, it is a laborious technique which may not readily be used in clinical or reference laboratories. Most of the markers currently being used as predictors of outcome are used in immunohistochemistry where tissues are thinly cut onto slides and stained with various antibodies. For that purpose it was imperative that we also develop an antibody which could be readily used in immunohistochemical analysis. During year 3 of this application we have worked

on developing a polyclonal antisera to cyclin E which shows preferential binding to the lower molecular weight isoforms of cyclin E protein. We have used this antibody in immunohistochemical analysis of cyclin E using frozen tissue sections left over from Western blot analysis. A representative staining of cyclin E is shown in figure 5. This figure clearly demonstrates that our antibody can differentiate between normal adjacent and tumor tissue from the same patient. Breast cancer tissues were stained with the polyclonal antibody to cyclin E by the peroxidase technique and counter stained for DNA with Ethylene Green. The immunohistochemistry shows high levels of cyclin E protein in high stage infiltrating ductal carcinoma while little to no cyclin E staining is observed in normal adjacent tissues (Fig 5). Our initial analysis correlating the levels of expression of cyclin E using Western blot analysis is comparative to the immunohistochemistry performed on 150 tissues .

Identify the multiple isoforms of cyclin E protein in tumor cells. In order to identify the isoforms of cyclin E protein a series of 5 multi-antigenic (MAP) peptides were generated. The 5 peptides are 30 AA each and are sequential starting at the carboxy-terminus. These peptides have been used to generate polyclonal antisera which cross-reacts specifically with cyclin E. One of these polyclonal antisera (to the carboxy-terminal 30 AA MAP peptide) shows preferential binding to the low molecular weight isoforms of cyclin E protein and was used in immunohistochemical analysis of cyclin E in tumor tissue samples presented in figure 5. This polyclonal antisera has been affinity purified and used to create an immuno-affinity purification column. We present preliminary data on the immuno-affinity purification of cyclin E (Fig 6). As shown, the column is able to preferentially bind the low molecular weight species of cyclin E while the largest species elutes during the column washes. This column has been very useful in purifying cyclin E which will be used for protein sequencing and mass spec determinations. By purifying and sequencing the lower molecular weight isoforms of cyclin E protein we will be able to understand the molecular mechanism leading to the alteration of cyclin E in tumor cells and help to identify those species which are preferentially being expressed in tumor but not normal cells.

Investigate the mechanism of deregulation of cyclin E in breast cancer cells. During the second year of this grant we examined the functional redundancy of cyclin E in tumor cells. Cyclin E is an important regulator of cell cycle progression that together with cyclin-dependent kinase 2 (cdk2) is crucial for the G1/S transition during the mammalian cell cycle. We showed that severe overexpression of cyclin E protein in tumor cells and tissues results in the appearance of lower molecular weight isoforms of cyclin E which together with cdk2 can form a kinase complex active throughout the cell cycle. We have found that one of the substrates of this constitutively active cyclin E/cdk2 complex is pRb in populations of breast cancer cells and tissues which also overexpress p16. In these tumor cells and tissues, we show the expression of p16 and pRb are not mutually exclusive. Overexpression of p16 in these cells results in sequestering of cdk4 and cdk6, rendering cyclin D1/cdk complexes inactive. However pRb appears to be phosphorylated through out the cell cycle following an initial lag revealing a time course similar to phosphorylation of GST-Rb by cyclin E immunoprecipitates prepared from these synchronized cells. Hence, cyclin E kinase complexes can function redundantly and replace the loss of cyclin D-dependent kinase complexes which functionally inactivate pRb. In addition the constitutively overexpressed cyclin E is also the predominant cyclin found in p107/E2F complexes throughout the tumor but not the normal cell cycle. These observations suggest that overexpression of cyclin E in tumor cells which also overexpress p16, can bypass the cyclin D/cdk4-cdk6/p16/pRB feedback loop, providing yet another mechanism by which tumors can gain a growth advantage. The results summarized in this section have been already published [75] and included in the Appendix section of this report.

During the third year of this application we have further probed into the molecular mechanism of deregulation of cyclin E in tumor versus normal cells by using the ribonuclease protection assay to identify the relative levels of cyclin E and its variants in normal versus tumor cells. The rationale for using this strategy to identify the lower molecular weight isoforms of cyclin E is that we observed a distinct heterogeneity of cyclin E mRNA in RT-PCR assays using normal versus tumor cell RNA as templates (Fig 7A) RT PCR shows that the mRNA from the tumor cell line MDA MB

157 is not composed of a single species. A series of nested oligos which start at the 5' end of the cDNA, make sequentially shorter PCR products. The normal cell strain 76N generates single PCR products with each set of nested primers. However, the tumor cell line MDA MB 157 shows multiple PCR products in the 5' end of the cDNA which shows that heterogeneity exists in this part of the mRNA. Some alternate forms of cyclin E mRNA have already been described and are shown graphically figure 7B. Other published forms include a 15 amino acid addition at the amino-terminus and a deletion in the cyclin box [77].

In order to identify the relative levels of cyclin E and its variants in normal versus tumor cells we have used the sensitive Ribonuclease Protection Assay (RNase) which is 10-50 times more sensitive than Northern Blot analysis. RNase protection probes were designed to examine cyclin E mRNA. To investigate the identity of the variant forms of cyclin E found in tumor RNA we designed several RNase protection probes Four overlapping antisense probes were used to quantitate the coding domain of the message as well as scan for splice variants. An example of one probe (of four different probes used) is shown (Fig 8A) demonstrating a typical standard curve and comparing the levels of mRNA in MCF 7 and MDA MB 157. The results of all four probes which scan the full length of the cyclin E mRNA are shown graphically (Fig 8B). Interestingly, one segment in the cyclin box domain is significantly over-represented (probe 3B). When a larger probe is used in the RNAse protection assay (probe 7B) two bands are clearly detected, which supports the presence of the over-represented version of cyclin E mRNA (Fig 9). This segment is now being cloned and sequenced by RACE RT PCR.

The quantitation of cyclin E mRNA was accomplished in seven cell lines including two derived from normal tissue and five derived from tumor tissue (Fig 10). The results of the quantitation showed that there are 2-3 copies of cyclin E mRNA per cell in unsynchronized normal breast epithelial cells (76N) and a normal breast cell line (MCF 10A). Breast tumor cell lines exhibited a range of cyclin E mRNA expression from as low as 1 copy per cell (MCF 7 and ZR75T) to four

copies per cell (MDA MB 231) to 8 copies per cell (MDA MB 436) to 40 copies per cell in MDA MB 157. Human beta actin was quantitated by RPA in each preparation of total RNA as a control. For comparison, beta actin was present consistently at about 3400 copies per cell regardless of cell type. This precise information regarding expression levels of cyclin E mRNA will be used later in anti-sense knockout/replacement experiments where the levels of cyclin E mRNA will be carefully regulated with a tetracycline inducible promoter.

Investigate the Oncogenic Potential of cyclin E and its interplay with p21: In the second year of this application we reported on the role of cyclin dependent kinase inhibitors in normal versus tumor cells and their cell cycle expression. We concluded that overall the cyclin dependent kinase inhibitors, in particular p21 is overexpressed in normal but not tumor cells. We further showed that via a pharmacological method, i.e. treatment of cells with Lovastatin, we were able to upregulate the CKIs in tumor cells and such induction lead to G1 arrest. These observations have now been published and we are enclosing a reprint of this manuscript in the Appendix. In the third year of this grant application we also have developed a model vector system to examine the oncogenic potential of cyclin E in normal cells and whether the overexpression of cyclin E will lead to the down-regulation of p21 in normal cells.

We are directly assessing the role of cyclin E in normal cells and breast tumor cell lines by a series of transient and stable transfection studies. Initially, we will tag cyclin E with a FLAG sequence and transfect into normal and tumor cells in an inducible vector. This system will allow us to differentiate the endogenous and exogenous (i.e. FLAG-tagged) cyclin E in normal cells and further evaluate whether such an overexpression can lead to either G1 arrest or alternatively a shortening of G1 in normal cells and increased transformability. Since constitutive overexpression of cyclin E may be toxic to cells, we have constructed the Flag-tagged cyclin E in an inducible vector which will be turned on by the removal of tetracycline from the medium as described in our grant application. Having constructed the vectors we are now in the process of transfection and

analyzing the derived phenotypes. The second approach which we are taking is to first knockout the cyclin E sequence from normal and tumor cells and then overexpress the different variant forms of cyclin E which we have previously identified and published on [78]. The utility of this approach is that we will first determine what the affects of knocking out of cyclin E be on maintaining cell cycle progression in normal versus tumor cells. Will the two types of cells respond differently to the absence of cyclin E? Will they both arrest? In addition we will be able to assess the impact of overexpression of the individual forms of cyclin E in normal versus tumor cells. In these experiments we will knock out the endogenous expression of cyclin E with an antisense sequence directed to the 5' untranslated region which spans a splice junction and the translation start codon. This antisense sequence is generated in the cell nucleus via a eukaryotic expression vector using green fluorescent protein (EGFP) as a reporter. The antisense sequence should encumber the primary transcript, promote degradation and prevent export from the nucleus. The antisense sequence has been designed to be physically separated from other plasmid RNA synthesized. This occurs by virtue of its unique placement after the polyadenylation sequence of the reporter gene. This insures high transcription rate driven by the powerful reporter gene promoter and detachment by the polyadenylation endonuclease activity. This free floating antisense sequence is available to bind the cyclin E primary transcript. The antisense sequence will block cyclin E expression and the transfected cells will arrest in late G1. Currently the vectors to accomplish this have been constructed and sequenced. They are capable of expressing EGFP as detected by flow cytometry. The optimal transfection conditions have been determined to achieve 50% transfection rates. Flow cytometry will used to examine DNA content on EGFP gated cells. The antisense will block cyclin E expression and the transfected cells will be arrested in G1 as determined by flow cytometry.

This analysis will not only examine the oncogenic potential of cyclin E as originally outlined in our grant application but also what the effects of knocking out of cyclin E is in these two cell types. The interplay with p21 will also be examined it he transfected cells as outlined in the original grant

application. We are in the midst of these experiments and anticipate completion of these aims in year 4 and 5 (we are asking for extension of this application).

In addition to the manuscripts on cyclin E redundancy (i.e. PNAS) and cyclin-dependent kinase regulation in normal versus tumor cells (Cancer Research) we also have written a very comprehensive review of cyclin E which is included in he appendix and will appear in a book chapter on cell cycle regulation to appear in 1997.

7: Conclusions:

The first Aim of our studies, use of cyclin E antibody as a diagnostic prognostic marker for breast cancer is now completed and the manuscript is currently being prepared. As outlined in this progress report we have surpassed our initial goal of collecting and extracting 150 tissue samples per year by increasing this number to 385 samples. (We have cyclin E data on over 500 samples but complete clinical and research data on he reported 385 samples).

The second Aim of the application deals with utilizing the deletional mutations of cyclin E to detect early metastatic breast cancer. We have documented [79] that these truncated forms of cyclin E are not deletional mutations and are in fact splicing variants of cyclin E found in normal and tumor cells and tissue samples. As outlined in the third aim of our application we have also investigated the regulation of cyclin E in normal versus tumor cells and found that cyclin E is deregulated in tumor cells. Such deregulation of cyclin E in tumor cells leads to its functional redundancy. As extension of the second aim of our study we have investigated the relative levels of cyclin E in normal versus tumor cells by the very sensitive method of RNAse protection assay and found that that although this transcript is present at only 1-3 copies in most normal cells and up to 40 copies per cell in tumor cells. Furthermore, these analysis revealed that there are indeed multiple isoforms of cyclin E present in both normal and tumor cells, yet only tin the tumor cells do these isoforms

give rise to protein products. For the studies outlined in the 3rd and 4th Specific Aims of our application we have already reported on the altered regulation of cyclin E and cyclin-dependent kinase inhibitors in tumor cells. We also have reported on a pharmacological (i.e. Lovastatin) method by which the cyclin-dependent kinase inhibitors can be overexpressed in tumor cells. Lastly, we have constructed the vectors required to perform the studies on the oncogenesity of cyclin E using two different approaches which will elucidate not only what affects will the overexpression of cyclin E have on the cell cycle progression normal cells, but what affects will its knocking out will have on both normal and tumor cells. It is anticipated that these studies will be completed by year 4 or year 5 (with extension) of this application. We will use the antisense expression vectors described above to assess loss/gain of function. In these transfections we will knock out the endogenous expression of cyclin E in normal breast cell strains and tumor cell lines. When we are sure the endogenous cyclin E is no longer expressed, we will substitute the endogenous cyclin E with wild type or splice variant forms of cyclin E. By this substitution we will assess the loss/gain of function of several easily measured phenotypic markers. Our hypothesis is that abnormally high levels of cyclin E/cyclin-dependent kinase activity (cdk 2) directly affects specific phenotypes found in tumor cells. These phenotypes include doubling time, ploidy, kinase inhibitor levels (p16 and p21), cyclin E levels and variant forms of cyclin E. These phenotypes are relatively easy to evaluate by standard laboratory techniques such as flow cytometry and Western blotting. We discovered these phenotypes while attempting to establish cloned tumor cell populations to be used for transfection experiments. It was hoped that a clonal background would reduce variation due to tumor cell line heterogeneity. We have found that these phenotypes define a clear heterogeneity found in a human breast tumor cell line MDA MB 157. When subclones of this cell line were analyzed, they differed significantly in the phenotypes described above. We currently have 70 unique cloned cell lines derived from the parent cell line MDA MB 157. We believe this heterogeneity is important in developing and/or maintaining tumor growth.

8. References

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Figure Legends

Figure 1: Demographic and Multivariate analysis of cyclin E. The population studied was a group of 385 patients who underwent surgery between 1990 and 1995 at 7 different hospitals in the Chicago area for breast cancer. Complete clinical and histopathological date for the multivariate analysis was available in all patients. Cyclin E expression was monitored both by Western blot and immunohistochemistry and Cyclin D 1 was analyzed by Western blot analysis using two different antibodies.

Figure 2: Cyclin E, a prognostic marker for breast cancer. Whole cell lysates were extracted from 12 breast cancer tissues, obtained from patients diagnosed with infiltrating ductal carcinoma. The Clinical staging, i.e TNM, is as indicated. Protein extracts were analyzed on Western blots (50 μg of protein extract/lane) and hybridized with the indicated antibodies. The control lanes correspond to cultured normal and tumor cell lines, where N= 76N normal cell strain and T=MDA-MB-157 tumor cell line.

Figure 3: Cyclin E, a better prognostic marker for breast cancer than cyclin D1. Cyclin E and cyclin D1 levels were examined by Western blot analysis on 385 breast cancer cases. High and low cyclin E and D1 levels were scored according to densitometric scanning and correlated to patient prognosis. Good prognosis is when the patient is alive and disease free, while poor prognosis is when the patient has either expired of the cancer or the cancer has metastasized or relapsed. NED: no evidence of disease.

Figure 4: Cyclin E is a Stage Independent Prognosticator for Breast Cancer. Cyclin E levels were examined by Western blot anlaysis on 385 breast cancer cases. Those tumor with high cyclin E levels (i.e 99) were analyzed according to the stage of the disease and correlated to patient prognosis. Stage of the disease is based on the clinical TNM staging. Good prognosis is when the

patient is alive and disease free, while poor prognosis is when the patient has either expired of the cancer or the cancer has metastasized or relapsed.

Figure 5: Analysis of cyclin E protein by immunohistochemistry. Breast cancer tissues (top panels tumor, bottom panels normal adjacent tissues) were stained with a polyclonal antibody to cyclin E by the peroxidase technique (DAB, Chromagen) and counter stained for DNA with Ethylene Green. Immunohistochemistry shows high levels of cyclin E protein in high stage IDC while little to no cyclin E staining is observed in normal adjacent tissues (X40).

Figure 6: Immuno-affinity purification of cyclin E. An anti-peptide polyclonal antibody to cyclin E was developed which could recognize all the lower molecular weight isoforms of cyclin E and used in immunohistochemical analysis of cyclin E in breast cancer patients (see figure 5). To characterize the specificity of this antibody, Cyclin E was purified on a polyclonal affinity column. The column was made by chemically crosslinking specific anti-peptide rabbit antibodies to protein A sepharose with DMP. MDA MB 157 cells were grown in culture and protein was prepared from the 100 k x g supernatant of sonicated cells. 8 mg of protein (400 microliters) were mixed with an equal volume of Buffer C, loaded into the affinity column and rotated overnight at 4 degrees C. The flow-through, subsequent washes and eluted cyclin E were collected and a portion of each was boiled in SDS PAGE sample buffer, separated by SDS PAGE, Western blotted and detected with the anti-cyclin E monoclonal HE12 using an HRP-labeled secondary and chemiluminescence. Wash #1, Buffer C, 50 mM Tris, pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.5% Nonidet P-40.Wash #2, Buffer A, 50 mM Tris, pH 8.0, 1 mM EDTA, 0.5 M NaCl, 0.5% Nonidet P-40.Wash #3, Buffer B, 50 mM Tris, pH 8.0, 1 mM EDTA, 0.15 M NaCl,0.1% SDS, 0.5% Nonidet P-40.Wash #4, Buffer C.Elution Buffer, 100 mM Glycine, pH 2.0 (neutralized with 1/5 volume 1 M Tris, pH 8).DMP, dimethyl pimelimidate.2HCl (Pierce)

Figure 7: Heterogeneity of Cyclin E mRNA: Total RNA was prepared from the normal cell strain 76N and MDA MB 157 by extraction with GIT buffer. The total RNA was purified by cesium chloride centrifugation and phenol/chloroform extraction. Specific oligonucleotide primers were used to generate PCR products using TAQ polymerase in 30 cycles. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. (B) Alternate forms of cyclin E mRNAare shown graphically.

Figure 8: Quantitation of cyclin E mRNA by Ribonuclease Protection Assay: (A) In order to quantitate the amount of cyclin E mRNA in MDA MB 157 an RNAse protection assay was used. An example of one probe (of four different probes used) is shown demonstrating a typical standard curve and comparing the levels of mRNA in MCF 7 and MDA MB 157. (B) The results of all four probes which scan the full length of the cyclin E mRNA are shown graphically. Interestingly, one segment in the cyclin box domain is significantly over-represented (probe 3B). The RNAse protection assay was performed using a probe derived from a PCR fragment of cyclin E which was ligated into the plasmid vector pCR 2.1. The T7 RNA polymerase promoter of the plasmid vector was used to generate a 32P labeled antisense RNA probe with the Maxiscript kit from Ambion, Inc. The probe was acrylamide gel purified and eluted prior to use. 20 micrograms of total RNA from MDA MB 157 was used to hybridize with an excess of radiolabeled antisense probe. The resulting hybridization products were RNAse digested and prepared using the RPA II kit from Ambion, Inc.

Figure 9: Ribonuclease Protection Assay with cyclin E probe p7B. When a larger probe is used in the RNAse protection assay (probe 7B) two bands are clearly detected, which supports the presence of the over-represented version of cyclin E mRNA. This segment is now being cloned and sequenced by RACE RT PCR. The presence of two bands which are protected by the larger probe 7B indicates that two species of mRNA exist. One species is capable of protecting the entire probe 7B while the other unknown species can protect only approximately half of the probe

7B.The RNAse protection assay was performed using a probe derived from a PCR fragment of cyclin E which was ligated into the plasmid vector pCR 2.1, as described in figure 8.

Figure 10: RPA Quantitation of cyclin E mRNA in normal and tumor breast cell lines: Cyclin E mRNA was quantitated in many different human breast cell strains and tumor cell lines utilizing the RNAse protection assay as described in figure 8 and a standard curve generated as described below. The copies per cell are quite low in most cell lines. The amount of cyclin E mRNA necessary to procede through cell division may be so low that cyclin E transfection experiments must be carefully designed to prevent over-expression.Quantitation was performed using the RNAse protection assay with a sense RNA to generate a standard curve. The sense RNA was prepared from a plasmid containing cyclin E cDNA in the sense orientation relative to the T7 promoter. Sense RNA was synthesized with the Maxiscript kit from Ambion, Inc. gel purified and quantitated by comparing ethidium bromide staining intensity to a known RNA standard.

Figure 1A

ID#	94-432
KK#	033
Gel #	TT003; TT013; TT047
Age	39
Diagnosis	Infiltrating ductal carcinoma
Tissue Type	Breast
Hospital	Swedish Covenant
ER status	0
PR status	17.23%; 78 fmoles/mg protein
DNA Index	1.44
Ploidy	Aneuploid
Proliferation Index	13.06 (high)
Clinical stage	3B: 4,1B2,0 (8/11 nodes positive)
Treatment	Radiation and chemotherapy
Outcome	Last contact 6/95, expired of cancer
Cyclin E by actin	2.46
Cyclin E, # of bands	6
Cyclin E, Total Dens.	1,235,876
Cyclin D1, mono -R-124	0
Cyclin D1, PRAD1	0
Cyclin E IHC, % positive	65.0 - 67.43
Cyclin E, IHC, OD/Pixel	0.213 - 0.22
Cyclin E, IHC Total:	14.30 - 14.36
Actin	502,526
PCNA	
Her 2 blot	
Her 2 stain	0.39 (positive)
p53	negative (see report)

Figure 1B

Cyclin E Breast Cancer Patient Population Demographics

Total Number of Cases Examined: 385

Year of Diagnosis: 1990-1995

			ER/PR	
			+ve	-ve
Age:	< 50	95 (25%)	57 (60%)	38 (40%)
	≥ 50	290 (75%)	290 (70%)	88 (30%)

Stage Groupings (i.e TNM)

I	113 (30%)
II	188 (49%)
III	55 (14%)
IV	29 (7.5%)

Figure 2

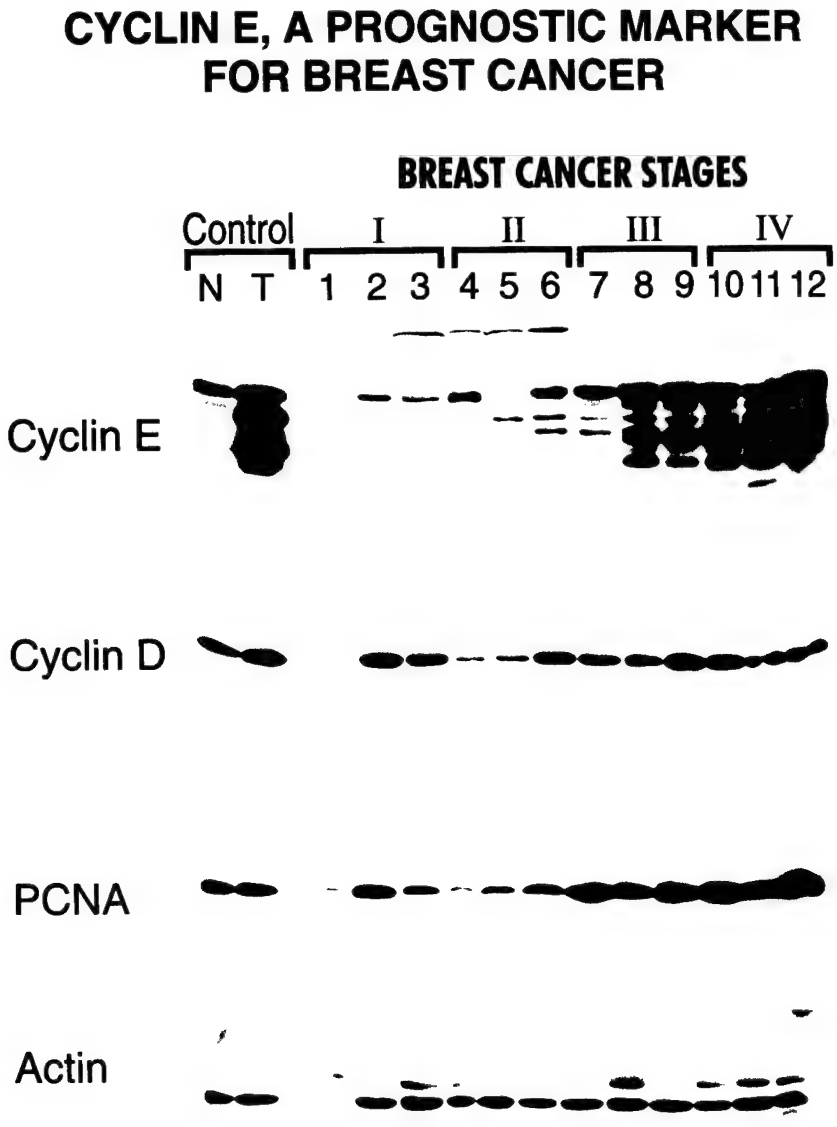
CYCLIN E, A PROGNOSTIC MARKER
FOR BREAST CANCER
BREAST CANCER STAGES
Control
I
II
III
IV
N T 1 2 3 4 5 6 7 8 9 10 11 12
Cyclin E
Cyclin D
PCNA
Actin

Figure 3

Cyclin E, A Better Prognostic Marker For Breast Cancer Than Cyclin D1

Total Cases	Low Cyclin E	High Cyclin E
385	286	99
Alive (NED)	260 (90%)	11 (11%)
Dead or with cancer	26 (10%)	88 (88%)

	Low Cyclin D1	High Cyclin D1
	198	187
Alive (NED)	153 (77%)	113 (60%)
Dead or with cancer	45 (23%)	74 (40%)

Figure 4

Cyclin E is a Stage Independent Prognosticator for Breast Cancer

Stage	Expired/ with Cancer	Alive (NED)
I	11	0
II	35	2
III	26	5
IV	16	4

Figure 5

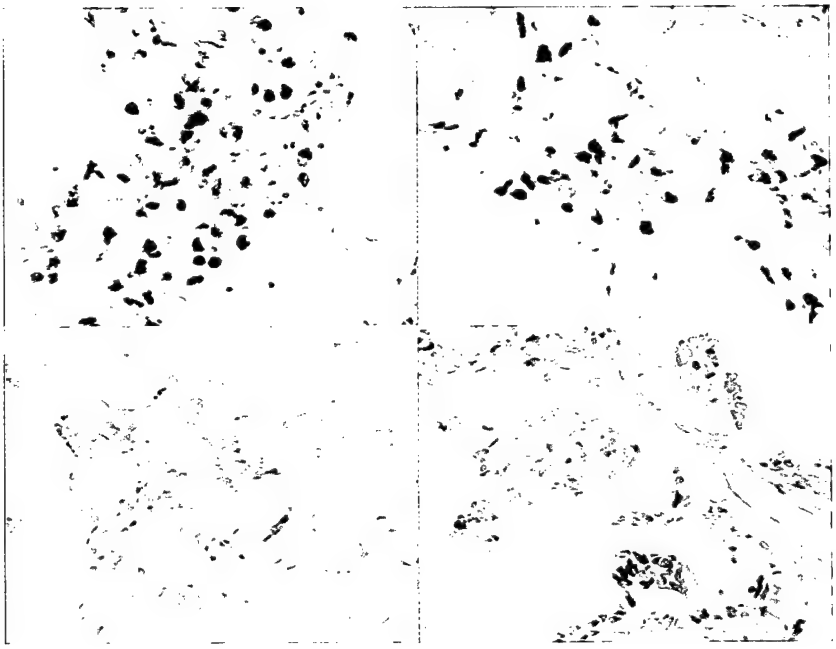

Figure 6

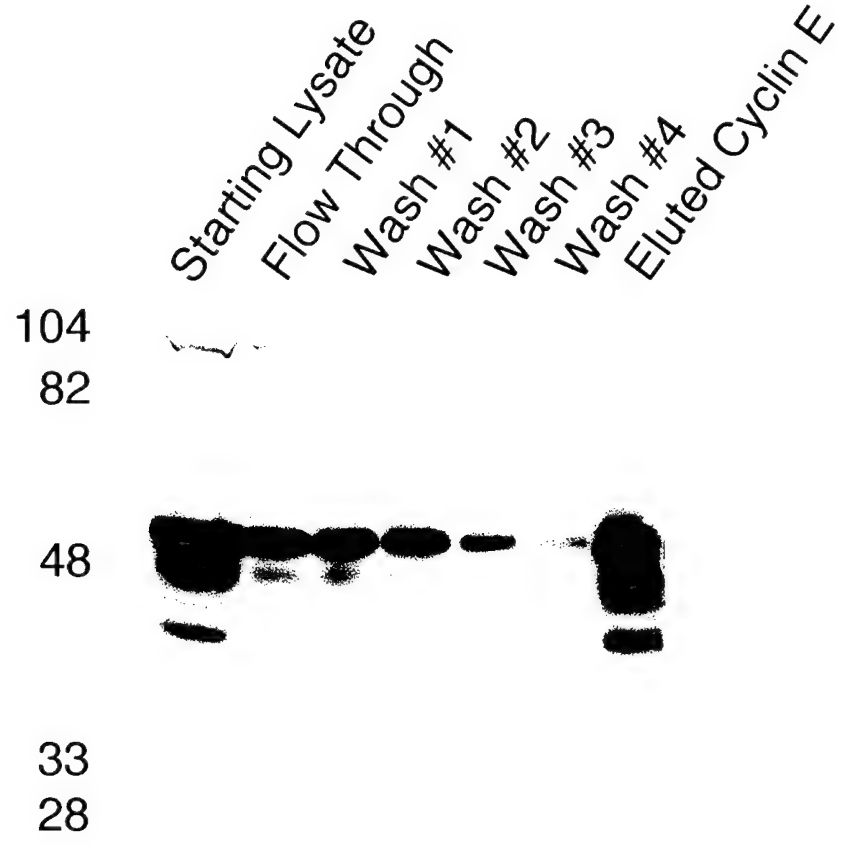
Immuno-affinity Purification of Cyclin E
Starting Lysate
Flow Through
Wash #1
Wash #2
Wash #3
Wash #4
Eluted Cyclin E
104
82
48
33
28

Figure 7

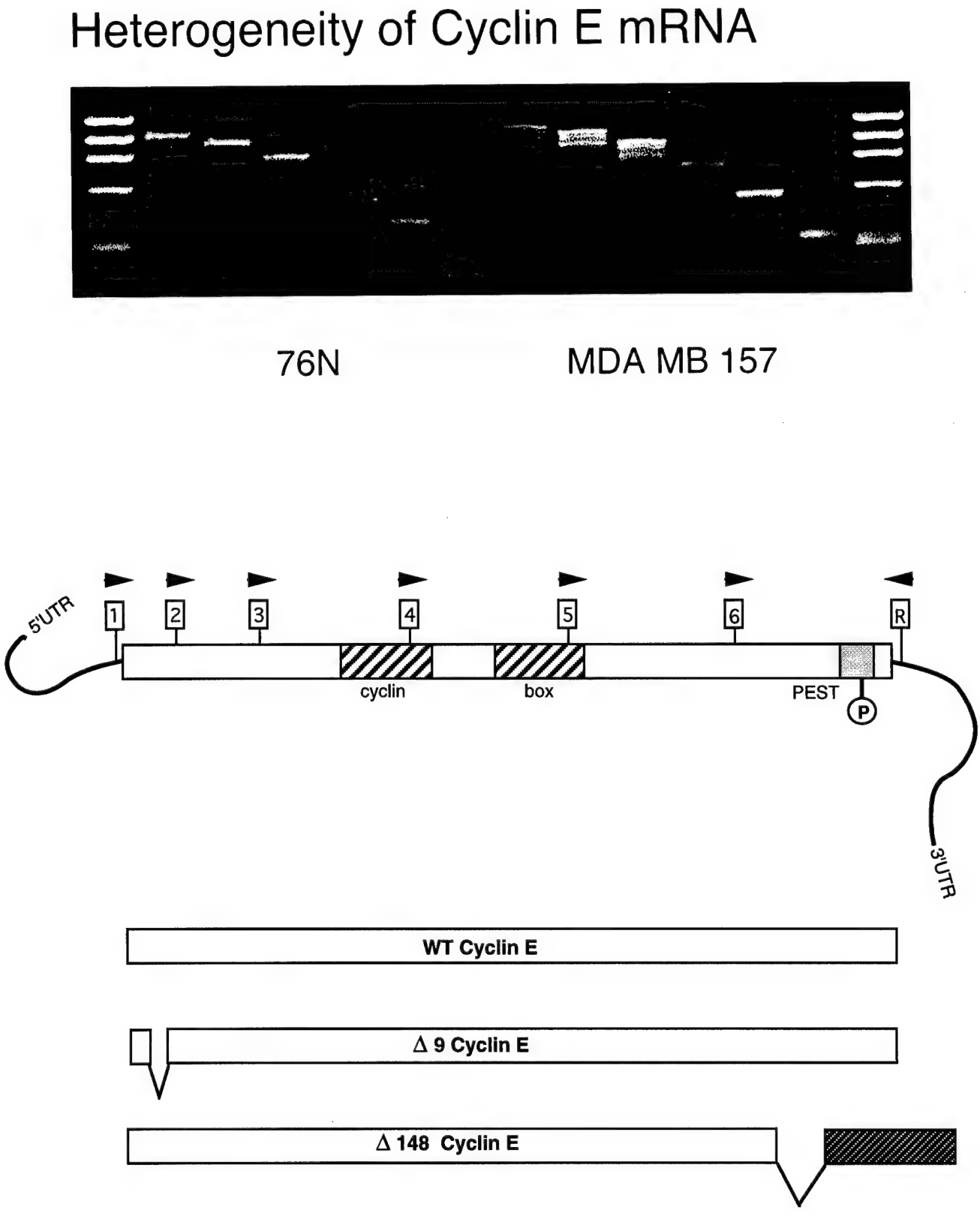
Heterogeneity of Cyclin E mRNA
76N
MDA MB 157
5'UTR
1
2
3
4
5
6
R
cyclin
box
PEST
P
3'UTR
WT Cyclin E
Δ 9 Cyclin E
Δ 148 Cyclin E

Figure 8A

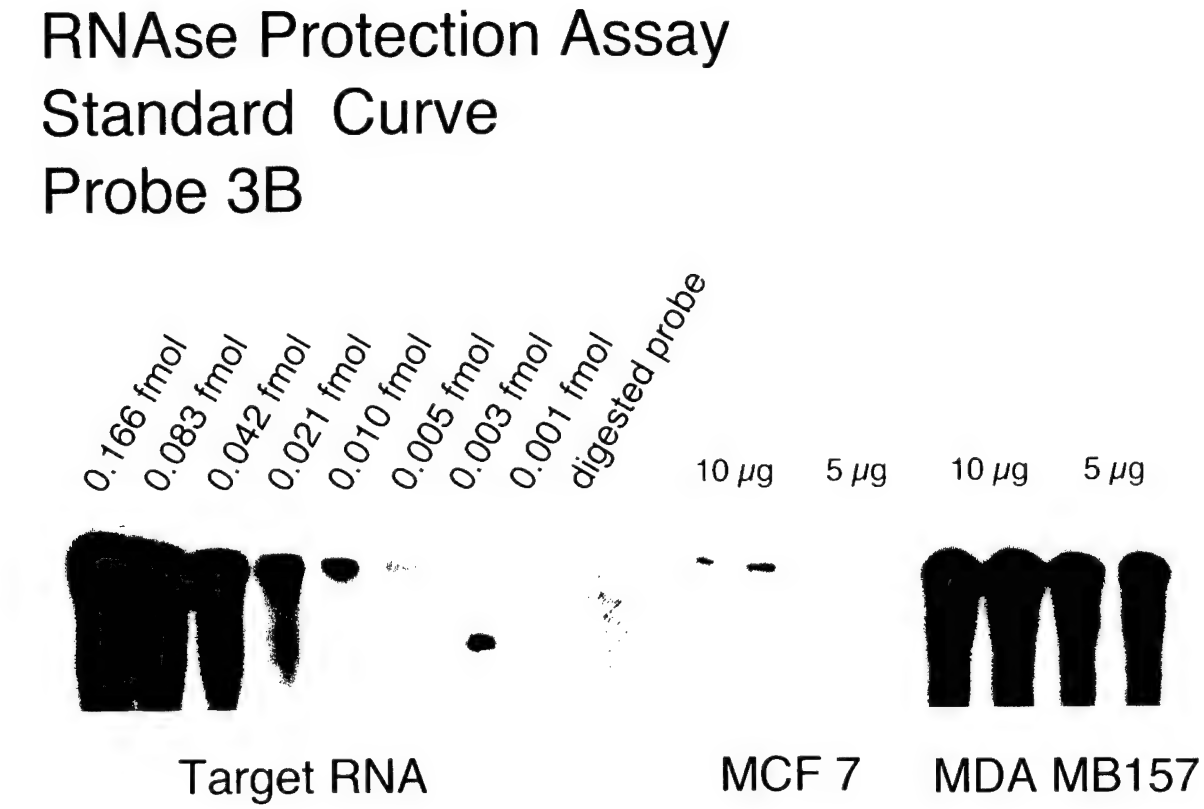
RNAse Protection Assay
Standard Curve
Probe 3B
0.166 fmol
0.083 fmol
0.042 fmol
0.021 fmol
0.010 fmol
0.005 fmol
0.003 fmol
0.001 fmol
digested probe
10 µg
5 µg
10 µg
5 µg
Target RNA
MCF 7
MDA MB157

Figure 8B

Quantitation of Cyclin E mRNA in MDA MB 157 by RPA

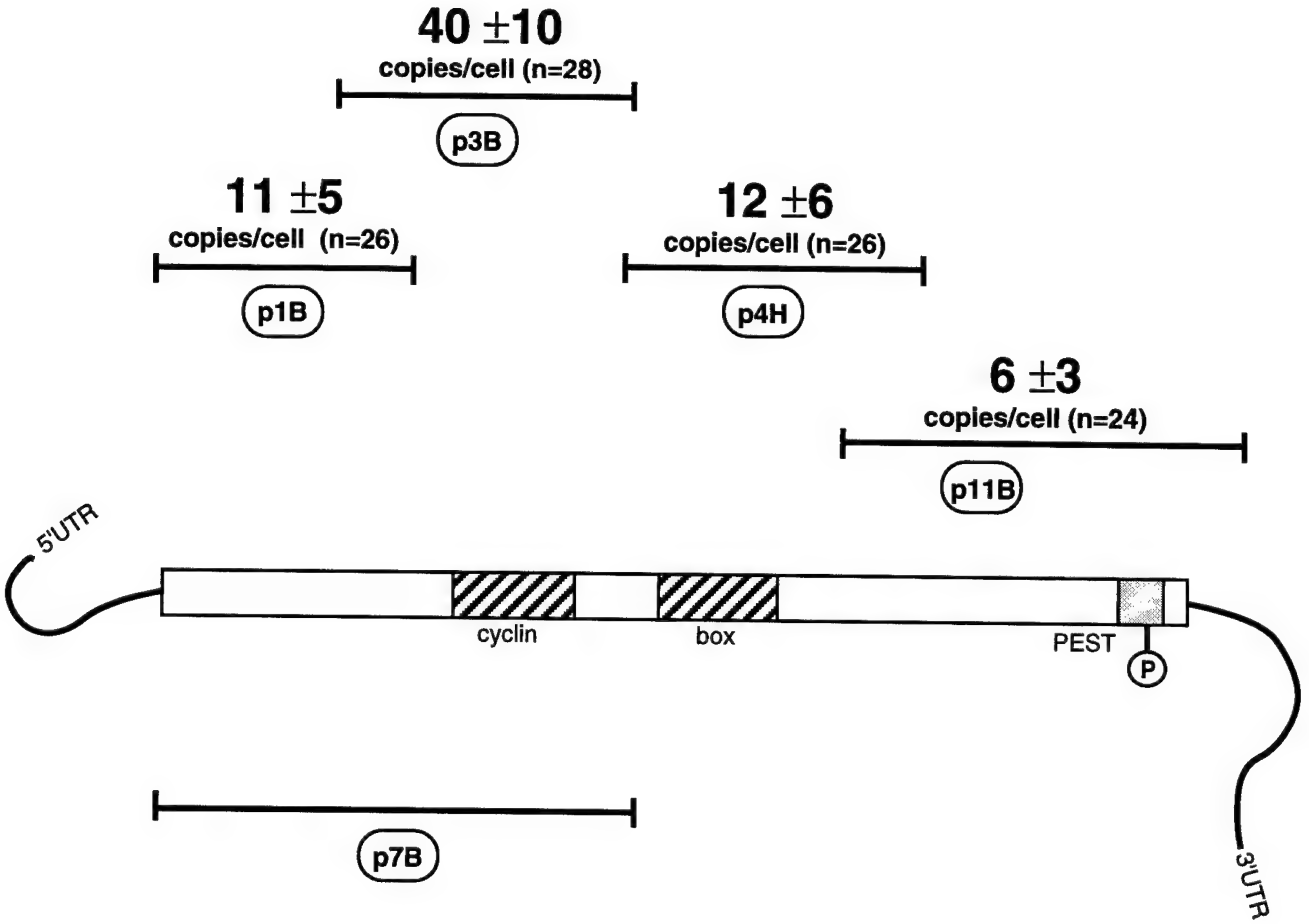

Figure 9

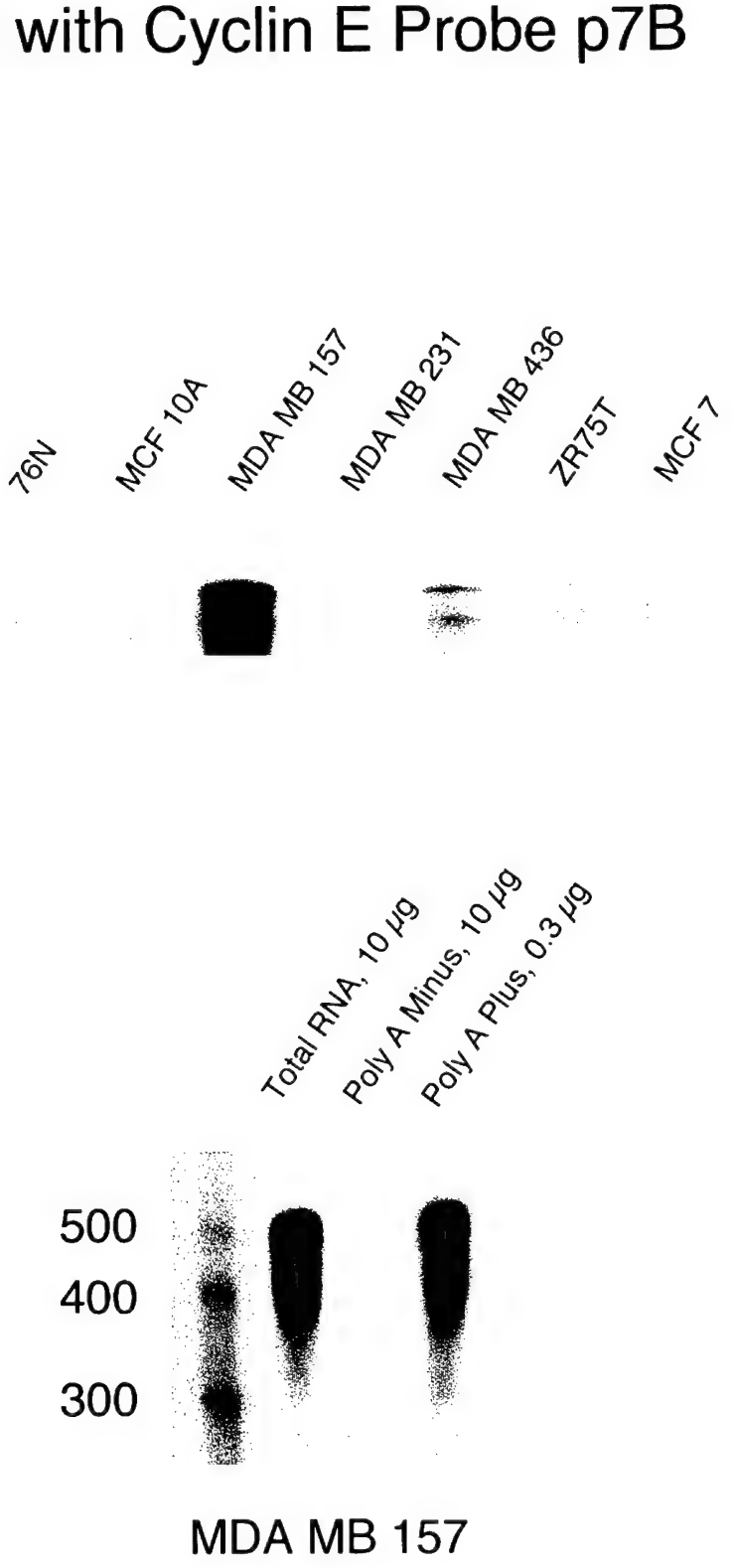
RPA with Cyclin E Probe p7B
76N
MCF 10A
MDA MB 157
MDA MB 231
MDA MB 436
ZR75T
MCF 7
Total RNA, 10 µg
Poly A Minus, 10 µg
Poly A Plus, 0.3 µg
500
400
300
MDA MB 157

Figure 10

RPA Quantitation of Cyclin E mRNA in Normal and Tumor Breast Cell Lines

	Probe 3B Copies per Cell	Actin Copies per Cell
76N	**3.1 ± 0.4**	**3028 ± 83**
MCF 10A	**2.2 ± 1.1**	**3657 ± 220**
MDA MB 157	**43.5 ± 2.1**	**3462 ± 206**
MDA MB 231	**3.6 ± 1.3**	**3484 ± 188**
MDA MB 436	**8.3 ± 1.4**	**5106 ± 327**
ZR75T	**0.9 ± 0.5**	**2321 ± 153**
MCF 7	**1.0 ± 0.6**	**1884 ± 102**

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(Meijer, L., Guidet, S. and Philippe, M., eds.) Plenum Press, New York, USA

chapter

The role of cyclin E in cell proliferation, development and cancer

Khandan Keyomarsi[1] and Thaddeus W. Herliczek

Wadsworth Center, New York State Department of Health,
Empire State Plaza, P.O. Box 509, Albany, NY 12201 USA.
[1] To whom correspondence should be addressed

Normal cell proliferation is under strict regulation governed by checkpoints located at distinct points in the cell cycle. The deregulation of these checkpoint events and the molecules associated with them may transform a normal cell into a cancer cell. One of these checkpoints whose deregulation results in transformation occurs at the Restriction point, near the G1/S boundary. The periodic appearance of one of the recently identified regulatory cyclins, cyclin E, coincides precisely with the timing of the Restriction point. The deregulation in the expression and activity of cyclin E has been associated with a number of cancers and is thought to be involved in the process of oncogenesis. In this chapter, we summarise the current knowledge on the regulation and apparent function of cyclin E in normal proliferating cells and in developing tissue and alterations of these processes in cancer.

CLONING OF CYCLIN E

Human cyclin E was discovered independently by two laboratories by virtue of its ability to rescue *Saccharomyces cerevisiae* cells lacking G1 cyclin function (1,2). The proteins encoded by the genes CLN1-3 (i.e. budding yeast G1 cyclins) are required for progression through START in *S. cerevisiae* (3-5). Yeast strains constructed with inactive CLN genes were grown on glucose, thereby repressing the GAL1 promoter and arresting the cells at START. Cells were then transformed with either HepG2 and HeLa (1) or human glioblastoma cell line U118 cDNA expression libraries (2). This approach was successful in identifying novel cyclins (i.e. cyclins C, D, and E) which encode proteins that could substitute for the yeast CLN proteins. However, in addition to the three novel G1 cyclins this strategy also identified non G1-type cyclins, such as cyclins A and B, which were capable of rescuing the CLN1-3 mutants. Subsequently, only cyclins D and E have been shown to have G1 function in higher eukaryotes. Following its cloning, several key questions could be answered regarding the role of cyclin E in the regulation of G1/S transition in normal cell proliferation, development, senescence and cancer.

CYCLIN E AS A REGULATOR OF G1/S TRANSITION

Several lines of experimentation provided ample evidence for the importance of cyclin E in G1/S progression in higher eukaryotes. Initially investigators demonstrated that cyclin E's mRNA and protein levels oscillated during the cell cycle accumulating in late G1 of synchronised human cells and declining as cells progress through S, G2 and mitosis (1,6,7). The cyclin E associated kinase activity also fluctuates periodically during the cell cycle with maximal activity at late G1 and early S phases, before the appearance of cyclin A. The cyclin dependent kinase (CDK) partner of cyclin E is CDK2. Immunoprecipitation of cyclin E followed by Western blotting with anti-CDK2 antibodies demonstrated that the relative levels of cyclin E-CDK2 complexes correlate with the cell cycle oscillation of cyclin E-associated kinase activity (6). Thus, cyclin E forms a complex exclusively with CDK2 and activates this serine-threonine kinase at the restriction point shortly prior to entry into S phase (8).

The ectopic expression of cyclin E into different cell lines also provided evidence for its role in G1 to S progression (9-11). Cyclin E overexpression resulted in a 3-5 fold increase in cyclin E-associated kinase activity in Rat-1 fibroblasts and primary human diploid fibroblasts using a retroviral expression vector. Transformed cells in which cyclin E levels and activity were constitutive throughout the cell cycle exhibited an altered cell cycle distribution consistent with a shorter G1 phase (9,12). Acceleration of G1/S transition by the overexpression of cyclin E was also observed in Rat-1 and HeLa cells using a tetracycline inducible system (10,11). In these studies, induced expression of cyclin E resulted in shortened G1 phase by 1.5-1.8 hours without significantly altering the total length of the population doubling time of the cells. In addition to shortening of G1 phase, the ectopic overexpression of cyclin E also resulted in a

decreased cell size and reduced the dependency of cells on growth factors and mitogens (9,11,12). Collectively these results support the regulatory role of cyclin E in G1 to S transition.

Since overexpression of cyclin E was shown to shorten G1, the inhibition of cyclin E expression and/or activity should prevent cells from progressing to S phase. Initially investigators studied the effects of inhibition of CDK2 (the catalytic activator of cyclin E) function in experiments where affinity purified anti-CDK2 antibodies were micro-injected into quiescent human diploid fibroblasts before and after the restimulation of cells by the addition of serum (13,14). These studies documented that the micro-injection of anti-CDK2, but not of anti-CDK1 antibodies prior to DNA synthesis blocked the entry of cells into S phase as measured by the ability of cells to incorporate [^{3}H]-thymidine (13) or BrdU (14), suggesting that CDK2 is required for the initiation of DNA synthesis. In separate studies performed to elucidate whether different CDKs have distinct roles in cell cycle control, investigators transfected dominant negative mutants of CDK2 into four different human cell lines: U2OS and Saos-2 osteosarcoma cells, C33A cervical carcinoma cells, and T98G glioblastoma cells (15). Expression of mutant CDK2 altered the cell cycle distribution in all cell lines examined resulting in a large increase in the G1 population. Furthermore, the effect of the mutant kinase was overcome by the cotransfection of a plasmid expressing the wild-type CDK2. Even though this suggests that CDK2 plays an essential role in G1/S progression, it was not shown which cyclin was required for this activity. Subsequent studies using affinity purified anti-cyclin E antibodies micro-injected into normal human fibroblasts proved that once cyclin E function is altered, entry into S phase is also blocked (12). In these studies, cells were arrested in G0/G1 by serum deprivation, micro-injected with anti-cyclin E antibody shortly after reactivation with serum, and the rate of DNA synthesis was measured by BrdU staining. These studies showed for the first time that micro-injection of anti-cyclin E antibody into G1 phase cells inhibited DNA synthesis. The time at which cyclin E function is required for entry into S phase was also determined by micro-injection of anti-cyclin E antibody at several time intervals following the restimulation of quiescent cells with serum. These studies determined that DNA synthesis initiated 12-16 hours after serum addition in non-injected control cells, while a significant inhibition of DNA synthesis was observed for up to 8 hours post restimulation in cells micro-injected with cyclin E antibody (12). Collectively these studies suggest that cyclin E has a major role in the G1 phase of the cell cycle prior to, but not during, S phase.

CYCLIN E AND DNA REPLICATION

The cyclin E/CDK2 complex also participates directly in DNA replication (16). This hypothesis is supported by several lines of evidence. In *Xenopus* eggs, which contain large maternal pools of cyclin E protein, DNA replication cannot proceed in the absence of this cyclin (17). When *Xenopus* cell extracts are: immunodepleted of cyclin E; incubated in the presence of the cyclin-dependent kinase inhibitor p21; or incubated with purified anti-cyclin E antibodies, chromosomal replication was ~~initiated~~ blocked. Hence, the cyclin E/CDK2 complex is essential for the earliest steps of chromosomal DNA replication including initiation or the switch to elongation in *Xenopus* eggs. This could be explained by either promoting the recruitment of replication proteins to the origins of DNA replication or by unwinding the DNA. In *Drosophila* embryos homozygous for a cyclin E mutation, cells arrest in the G1 phase of cycle 17, the first embryonic cell cycle to contain a G1 phase (18). In addition, the ectopic expression of cyclin E induces an extra S phase in *Drosophila* embryonic cells that had exited the cell cycle. Recently the factors that trigger the initiation of DNA replication in somatic cell nuclei were analysed in a human (HeLa) cell-free system (19). These analyses revealed that nuclei prepared from G1 phase, but not from G2 phase of HeLa cells are required to initiate semiconservative DNA replication. These nuclear factors can be replaced by human cyclin A and E when complexed with CDK2 to trigger initiation of DNA replication *in vitro*. Since cyclin E/CDK2 acted synergistically with cyclin A/CDK2 in the initiation of DNA replication, it is hypothesised that the two cyclin/CDK2 complexes act through separate pathways with active cyclin E/CDK2 preceding cyclin A/CDK2 in triggering the G1-S transition and the initiation of DNA replication (19). Lastly, the ectopic expression of human cyclin E, but not cyclin D1, induced a dramatic synthesis of DNA in *S. cerevisiae* cells under conditions of cell cycle arrest in START at G1 or G2/M, thereby uncoupling DNA replication from cell cycle progression (20).

CYCLIN E AND CELLULAR SENESCENCE

Aside from its role in the regulation of G1/S transition, cyclin E has also been implicated in the senescence of human diploid fibroblasts (21-23). The state of cellular senescence is characterised by an irreversible arrest of cells in the G1 phase of the cell cycle rendering the cells unable to enter S phase upon mitogenic stimulation. Several laboratories observed an abundance of cyclin E and D1 mRNA

and protein in fibroblasts undergoing cellular senescence. Even though there is a 10-15 fold overexpression of cyclin E and D1 in senescent compared to quiescent early-passage fibroblasts, the cyclin E-associated kinase activity in these cells is very low and does not increase upon mitogenic stimulation (21,23,24). The low kinase activity was not due to lack of complex formation with CDK2 but due to the underphosphorylated inactive forms of cyclin E/CDK2 complexes. Lastly, the inactive cyclin E/CDK2 complexes from senescent cells were not activated by cdc25 phosphatase *in vitro*, suggesting that the inactivity of cyclin E/CDK2 complexes in these cells is not due to inhibitory phosphorylation of CDK2 on Tyr-15 and Thr-14 residues. The inactivation of cyclin E/CDK2 complexes in senescent cells will render pRb underphosphorylated and result in the arrest of cells in late G1 phase, unable to proceed into S phase. This hypothesis is further corroborated by the identification of p21, a cyclin dependent kinase inhibitor, which was originally cloned due to its elevated expression in senescent cells (25). p21 (also known as cip1, WAF1, Sdi1, CAP20, and PIC1) may initiate an early step in cellular senescence by binding to and inactivating cyclin E/CDK2 complexes, giving rise to a terminally late G1 arrested cell (26). In this progression toward senescence, p21 forms a complex with E2F containing pRb, CDK2 and cyclin E, which will inhibit the E2F dependent transcription of genes critical for entry into S phase. Although cyclin E is overexpressed in senescing cells the function of this overexpression remains to be elucidated.

FACTORS REGULATING CYCLIN E/CDK2 EXPRESSION AND ACTIVITY

Transcriptional regulation of cyclin E

The expression, activation, and degradation of cyclin E are controlled at multiple levels in eukaryotes. The regulatory mechanisms mediating the up-regulation of cyclin E mRNA at the G1/S border were examined upon identification and characterisation of sequences containing the cyclin E promoter (27,28). These analyses revealed that the cyclin E promoter region contains several transcription factor binding sites, including six E2F and eleven Sp1 binding sites. The presence of multiple E2F binding sites suggested the involvement of the E2F transcription factor for the up-regulation of cyclin E mRNA in late G1. In fact when rat embryo fibroblasts (REF52) were transfected with plasmid containing the cyclin E promoter cloned upstream of a CAT reporter gene, starved for serum and then infected with a recombinant andenovirus constructed to overexpress E2F1, cyclin E promoter activity was stimulated by close to 200 fold (27). Similarly cyclin E promoter constructs were generated to contain a series of truncated and/or mutated derivatives of the promoter region eliminating the multiple E2F recognition sites and then assayed for E2F1-mediated activation (27,28). These analyses revealed substantial reduction in the response to E2F1 by mutating or eliminating the first three E2F sites, suggesting that the up-regulation of cyclin E expression is mediated by E2F's transactivation of the promoter. The role of retinoblastoma protein (pRb) in the E2F mediated up-regulation of cyclin E was also investigated by deciphering the association of mutant pRb with wildtype versus mutant forms of E2F in cells co-transfected with the cyclin E promoter. These analyses revealed that the mutant pRb inhibited the expression of cyclin E promoter by 7 fold in the presence of overexpressed wildtype E2F1. However co-expression of mutant E2F with mutant pRb reduced the expression of cyclin E promoter by only 1.4 fold. Collectively these analyses suggest that pRb negatively regulates the activity of cyclin E promoter through its association with E2F. Furthermore, these studies provide a model for the regulation of pRb phosphorylation by cyclin/CDK complexes active in the G1 phase of the cell cycle. In early G1, pRb, mainly present in its hypophosphorylated forms, binds and sequesters E2F, leaving little free E2F available to transactivate cyclin E. However, if pRb is not present, ~~in cells~~ levels of cyclin E are high and often deregulated (29). As cells progress through G1, cyclin D1 in complex with CDK4 or CDK6 can phosphorylate pRb and release E2F. Free E2F up-regulates cyclin E levels which in turn, complex with CDK2 and further phosphorylate pRb ~~and would~~ creating a positive feedback loop between inhibition of pRb and release of E2F for maintaining a high level of cyclin E expression required for G1/S transition (Figure 4A). This feedback loop could be limited by the action of ubiquitin mediated proteolysis of cyclin E (see below).

Phosphorylation and ubiquitination of cyclin E

Once cyclin E protein is expressed, it binds and forms a complex with CDK2. This complex is positively and negatively regulated by phosphorylation. The activating phosphorylation of this complex is achieved by another cyclin/CDK complex termed cyclin/CDK-activating kinase or CAK, which consists of cyclin H/CDK7 kinase. CAK activates cyclin E/CDK2 complex by phosphorylation of the conserved Thr-160 (30,31). Even though cyclin E can bind effectively to CDK2 in the absence of any phosphorylation, the activity of this complex is dependent on phosphorylation of Thr-160 (32). The Thr-160 phosphorylated forms of CDK2 are in complex with cyclin E and are capable of phosphorylating histone H1 *in vitro* (18,33). Cdc25A activates CDK2 by removing the inhibitory

phosphate groups on Thr-14 and Tyr-15 (34). Interestingly, cdc25A itself undergoes phosphorylation and activation in S phase which is dependent on the activity of cyclin E/CDK2 complex. In fact, CDK2/cyclin E complexes can directly phosphorylate recombinant glutothione S-Transferase tagged cdc25A *in vitro* and cdc25A stably associated with CDK2 and cyclin E *in vivo*. Furthermore, when a human diploid fibroblast cell line (IMR-90) arrested in G1 are micro-injected with antibodies to cdc25A, entry into S phase is blocked (35). These studies suggest that a positive feedback loop exists between activation of cyclin E/CDK2 complex and cdc25A phosphatase and the transition from G1 to S phase.

The periodic pattern of expression of cyclin E in the normal cell cycle is not only dependent on the rate of synthesis of the protein but also on its rate of degradation. Recently it was demonstrated that cyclin E degradation is mediated by ubiquitin-proteasome system and is dependent on CDK2 binding and cyclin E-CDK2 catalytic activity as well as site-directed phosphorylation of cyclin E itself (36,37). The ubiquitination and proteasomal degradation of cyclin E occurs when it is not bound to CDK2. This pathway is inhibited by the addition of the peptide aldehyde LLnL which inhibits proteasome-mediated proteolysis and leads to the accumulation of cyclin E. The association of cyclin E with CDK2 makes cyclin E resistant to ubiquitin mediated proteolysis and resistant to further accumulation by LLnL, ~~when cyclin E is bound to CDK2.~~ Furthermore, the auto-phosphorylation of cyclin E on Thr-380 promotes its ubiquitination and proteasome-dependent degradation, presumably by enhancing its dissociation from the cyclin/CDK2 complex and making it more susceptible to the ubiquitin-conjugating enzymes.

Negative regulation mediated by CKIs

An additional level of regulation of cyclin E expression and activity has become apparent with the discovery of cyclin dependent kinase inhibitors (CKIs), p21 $^{cip1/Waf1}$ and p27 ^{Kip1}. The CKI p21 was simultaneously characterised in several laboratories as the major p53 inducible gene (WAF1) (38-40), as a CDK inhibitor protein (CIP1, p21, and p20^{CAP1}) (34,41,42), as a protein highly expressed in senescent fibroblasts (sdi) (25), and as a melanoma differentiation associated gene (mda6) (43). In normal fibroblasts, this protein has been shown to be associated with various cyclin-CDK complexes, including CDK2 associated with cyclins A and E, CDK4 associated with D-type cyclins (41,42,44-46), and is also found weakly associated with cdc2-cyclin B (44). p21 can inhibit the kinase activity of CDK2, CDK4 and cdc2 cyclin complexes *in vitro* and *in vivo* as demonstrated in transgenic mice overexpressing p21 specifically in hepatocytes (47). p53 induction of p21 in response to DNA damage presumably results in CDK inhibition and G1 growth arrest (48). p21 may therefore be a crucial component in the DNA damage surveillance function of the tumor suppressor p53 (49). p21 can also be induced by p53 independent mechanisms; by serum, PDGF and EGF in embryonic fibroblasts from p53 knockout mice (50), by serum starvation in p53 mutant human breast carcinoma cells (51) and by TGF-ß1 in human ovarian cancer cells (52). Additionally, p21 can be induced during the differentiation of various cell types independent of p53 status: p21 is induced in HL60 leukemia cells by the differentiative agents vitamin D3 (53); 12-0 tetradecanoyl phorbol-13 acetate; retinoic acid; dimethlysulfoxide (54,55); butyrate (54); in melanoma cells induced to terminally differentiate with interferon and mezerein (43); and in K563 chronic mylogenous leukemia cells and U973 monocytic leukemia cells by okadaic acid (56). More recently p21 has been shown to be induced by the helix-loop-helix transcription factor MyoD and is associated with muscle differentiation *in vivo* (57). p21 was also found to be differentially expressed in other developing tissues and its expression correlated with terminal differentiation in a p53 independent manner (58). These observations suggest that p21 may be an important mediator of differentiative and/or anti-proliferative signals. Indeed the induction of p21 along with transcription factors *fos* and *jun* appears to be an immediate early response to differentiative signals unaffected or actually enhanced by the action of cyclohexamide (54). These observations suggest that p21 may directly affect downstream differentiation events.

Of the inhibitors identified to date, p27^{KIP1} is most similar in amino acid sequence and inhibitory specificity to p21^{CIP1}. p27^{KIP1} was identified simultaneously as a protein associated with inactive cyclin E-CDK2 complexes in TGF-ß1 treated and contact inhibited cells (59,60), as a protein that interacts with cyclin D1-CDK4 complexes (61), and in HeLa cells arrested in G_0 or by Lovastatin (62). TGF-ß1 arrests certain cell types in G1 and p27 is thought to be a cellular mediator for this anti-proliferative signal (63). Although p27 is not transcriptionally induced by TGF-ß1, it progressively accumulates in cyclin E-CDK2 complexes in TGF-ß1 treated cells (59,60). TGF-ß1 does, however, repress CDK4 expression which may result in displacement of p27 from cyclin D-CDK4 complexes rendering the CKI free to complex with and inhibit cyclin E-CDK2. Binding of p27 to cyclin-CDKs prevents the CAK mediated threonyl phosphorylation required for CDK activation (64). Additionally, a p27 protein has been found to be down-regulated in interleukin 2 induced proliferating T lymphocytes (65,66).

Hence, p21 and p27 may function similarly to inhibit CDK activity and proliferation in response to different environmental stimuli. Furthermore, removal by degradation or inactivation of these inhibitors may be necessary for proliferation.

Involvement of p21 and p27 in the regulation of cyclin E/CDK2 activity has been demonstrated in numerous systems. For example γ-irradiation of human diploid fibroblasts harboring wild type p53 in the G1 phase of the cell cycle results in the arrest of cells prior to S phase. Even though the irradiation of cells did not prevent cyclin E from accumulating, the activity of cyclin E/CDK2 and cyclin A/CDK2 complexes were significantly impaired. This inhibition of kinase activity in response to irradiation was dependent on p53 mediated induction of p21 (48). In a similar study performed independently using irradiated lymphoid cell lines in which p53 allele status has been characterised, p21 was detected in non-irradiated cells containing wildtype p53, but not in mutant p53 cell lines (38). Furthermore when the majority of the p53 wildtype lymphoid cell lines were induced to arrest in G1 by ionising irradiation, p21 was up-regulated, while no induction of p21 was observed in irradiated lymphoid cells with mutant p53. In addition, p21 induction was not observed in cells which undergo either G1 arrest or apoptosis through p53 independent pathways using either a glioblastoma cell line or a murine hematopoietic cell line. Lastly, the induction of p53 dependent p21 was directly correlated with the inhibition of cyclin E/CDK2 kinase activity which would subsequently lead to a G1 arrest.

A direct association of p21 and cyclin E/CDK2 was confirmed upon characterisation of p21 peptide domains required for cyclin E/CDK2 interactions (67). This analysis revealed that a synthetic peptide spanning amino acids 15-40 of p21 antagonised p21 binding and inhibition of cyclin E/CDK2 kinase activity. In addition p21 has been demonstrated to directly associate with cyclins, including cyclin E. The p21 amino acid sequence contains two copies of a cyclin-binding motif, one near the N terminus (CY1) and the other at the C-terminus (CY2), which interact with the cyclins independently of CDK2 (68). Disruption of CY1 either by mutation or antibody competition studies demonstrated that this cyclin binding motif of p21 is essential not only for the association of cyclins with p21, but also for the kinase inhibition and biological activity of p21 (68). In an independent study in which the nature of cyclin-CDK complex recognition with their substrates was examined, the same cyclin recognition motifs were identified and characterised in p21 (69). Both *in vitro* kinase binding and inhibition assays as well as *in vivo* growth suppression assays suggested that these two cyclin binding motifs are functional and essential for the inhibitory activity of p21 in cultured cells (69). Lastly, the p21 amino terminal cyclin binding motif seems to be conserved in all the p21 family members including p27 and p57, and mutation in residues within this cyclin binding motif eliminated the binding of cyclin D1, cyclin E, cyclin D/CDK4 and cyclin E/CDK2 to the protein (70).

The negative regulation of cyclin E by p27 was initially reported in studies where treatment of Mv1Lu lung epithelial cells with TGF-ß1 resulted in inhibition of cyclin E-CDK2 dependent kinase activity. The TGF-ß1 mediated inhibition of cyclin E activity was not due to inhibition of cyclin E or CDK2 protein synthesis because the levels of these proteins remained unchanged, but was due to the negative effects of TGF-ß1 on the assembly and subsequent phosphorylation of CDK2 (63,71). Subsequent studies demonstrated that TGF-ß1 up-regulated the binding of p27 to CDK2 which can inactivate cyclin E associated kinase activity and exert a negative regulation upon cyclin E (59,60,72).

While TGF-ß1 can inhibit the growth of epithelial cells in late G1 by inactivation of cyclin E-CDK2 complexes, it can stimulate growth in cell lines of mesenchymal origin, such as mouse fibroblasts. Interestingly p27 is the culprit in both cases[1]. For example treatment of epithelial cells by TGF-ß1 results in up-regulation of p27, while treatment of C3H 10T1/2 mouse fibroblasts by TGF-ß1 resulting in the induction of cell proliferation is associated with down regulation of p27 (74). The levels of p27 protein in complexes with cyclin E/CDK2 is significantly reduced in late G1, suggesting that TGF-ß1 can positively modulate the activity of cyclin E/CDK2 by decreasing the steady state levels of p27.

The inactivation of cyclin E-CDK2 in G1 by p27 also occurs when cells are treated with rapamycin or are arrested in G1 by cAMP. Rapamycin, a potent immunosuppressant, inhibits G1 to S transition in a variety of species. The mechanism of this G1 arrest is inactivation of cyclin E/CDK2 complexes by up-regulation of p27 binding to CDK2. In fact when T cells were mitogenically induced by IL-2 leading to activation of cyclin E/CDK2 complexes, p27 levels decreased significantly and addition of rapamycin inhibited this activation by increasing p27 levels (66). cAMP, an activator of protein kinase A, also

[1] The effect of TGF-ß1 on another epithelial cell line such as MCF-7 is also by inactivation of cyclin E/CDK2 complexes, not through overexpression of p27, but due to a three fold up-regulation of p21, which results in a time-dependent accumulation of p21 in the nucleus (72).

inhibits the growth of cells by preventing S phase entry through up-regulation of p27. Studies which initially documented the involvement of p27 in cAMP mediated G1 arrest were performed using macrophages (64). In these studies BAC1.2F5A macrophages were initially deprived of Colony Stimulating Factor 1 (CSF1) for 18 hours resulting in a G1 arrest and then stimulated to reenter the cell cycle in the presence of CSF-1 and activators of cAMP such as 8Br-cAMP. Such treatment prevented 80% of CSF-1 stimulated cells from entering S phase and resulted in the inhibition of cyclin D1/CDK4 kinase activity which is required for the phosphorylation of retinoblastoma. The inactivity of cyclin D/CDK4 complex was due to lack of its phosphorylation by cyclin-activating kinase (CAK) via the up-regulation of p27 which associates with cyclin D/CDK4 complex preventing CAK from phosphorylating and activating this complex (64). Similar conclusions were reached when the effects of cAMP on cell cycle progression of an astrocytic cell line were examined (75). Treatment of growth stimulated astrocytic cell line CLTT11 by Forskolin, an activator of adenylate cyclase, prevented cells from reinitiating DNA synthesis and was associated with decreased CDK2 associated H1 kinase activity presumably due to up-regulation of p27. Lastly, treatment of estrogen responsive breast cancer MCF-7 cells by anti-estrogens leads to a G1/S arrest, presumably as a consequence of reduced pRb phosphorylation mediated by lack of cyclin D1/CDK4 activity due to increase in p27 and/or p21 levels (76,77).

Cyclin E and cell anchorage

The regulation of cyclin E-CDK2 kinase activity is also dependent on cell anchorage. Cell anchorage or adhesion has been shown to have an essential role in regulating transition of G1 to S phase in non-malignant cells, while most transformed cells are anchorage independent. Initially investigators examined the dependence of cell anchorage on the activation of G1 cyclins along with their associated cyclin-dependent kinases in normal and transformed cells (78). When attached human fibroblast cells such as KD and IMR90 cells were synchronised in G_0 by serum deprivation and cultivated in suspended or attached cultures in the presence of serum, cells in suspension culture failed to enter S phase while attached cells progressed through S phase. Although the amounts of CDKs and cyclin E proteins were similar in the suspended and attached cultures, increased cyclin E/CDK2 activity was present only in the attached cultures. The lack of cyclin E-CDK2 activity in suspended cultures was associated with reduced phosphorylation on Thr-160 of CDK2 catalysed by CAK. The lack of cyclin E/CDK2 activity was subsequently attributed to increased levels of both p21 and p27 in suspended cultures which account for the decreased phosphorylation of CDK2 at Thr-160 (78). In another study using normal adhesion dependent versus Ha-ras transformed adhesion independent NRK fibroblast cells, cyclin E/CDK2 complex was also shown to be the target of the adhesion signal, since a catalytically active complex was formed only in adherent cultures. However, no increases in p21 and p27 were observed between adherent and suspended NRK cells. The authors attributed the decreased cyclin E/CDK2 activity in suspended cultures to a feed back loop requiring catalytically active CDK2 which promotes transcriptional activation of cyclin A required for G1 to S transition (79). In fact, a link between cyclin A expression and adhesion-dependent cell cycle progression was found in arrested NRK non-transformed cells where cyclin A is not expressed in response to loss of adhesion. However, the infection of NRK cells with a cyclin A retrovirus allowed for the anchorage-independent expression of cyclin A/CDK2 activity and resulted in anchorage independent growth of these infected cells (80). A subsequent study reported that loss of anchorage abrogates expression of cyclin A in NIH 3T3 cells at the transcriptional level (81). When NIH 3T3 cells are suspended, the activity of cyclin A gene promoter is blocked by inability of E2F to transcriptionally activate the cyclin A gene, the same mechanism responsible for the cyclin A transcription repression during G1 in adherent cells (82). Once adherent cells are serum stimulated, an activation specific complex containing p107 and cyclin E is formed which releases free E2F and promotes transcriptional activation of cyclin A. On the other hand, addition of serum to suspended cells does not result in the release of free E2F nor the formation of a cyclin E-p107 complex, but instead results in the stabilisation of p27 protein which is suggested to directly block the cyclin A transcription through an E2F-mediated pathway (81). The anchorage dependence of NIH 3T3 cells is also required for the phosphorylation of retinoblastoma protein and is associated with the anchorage dependent expression of cyclin D1 and activation of cyclin E/CDK2 complex (83). Collectively these observations lead to a model where the emergence of anchorage-independence occurs through a multi-step deregulation of adhesion controlled cell cycle events resulting in deregulated function of CDK complexes, specifically cyclin E/CDK2 holoenzyme required for G1 to S transition. Hence, as a consequence of its rate-limiting and essential role in G1 progression, cyclin E activity is a common downstream target of antimitogenic signals that block the cell cycle in G_0/G1. Thus cyclin E responds to a number of negative growth signals such as TGF-ß1, rapamycin, cAMP, irradiation, senescence, and

anchorage dependence inducing p21 and/or p27[2] which inhibit the cyclin E/CDK2 complexes.

Chemical inhibition of cyclin E/CDK2

Since inhibition of cyclin E/CDK2 ~~complex~~ under physiological conditions has been shown to lead to G1 arrest and cessation from entry in S phase in numerous systems, pharmacological induction of such an arrest in tumor cells would be quite beneficial and may compensate the loss of tumor suppressor genes and lack of checkpoint control. There are several agents which can induce either a G1 or G2 arrest or both by targeting either CDKs or other protein kinases. One such agent is the kinase inhibitor Staurosporine (ST) which can induce G1 and G2 arrest. When ST is used at low concentrations (2-20 nM) it arrests normal diploid fibroblasts in G1, while at higher concentrations (100-150nm) ST arrests these cells in both G1 and G2 (85-87). The mechanism of the ST mediated G1 arrest in normal human fibroblasts cells is thought to involve inhibition of pRb phosphorylation and CDK2 activity. Yet, treatment of cells with 20 nM ST did not inhibit pRb phosphorylation or CDK2 activity in tumor cells (i.e. HeLa) (88). The timing of ST action has been mapped to a period in G1 subsequent to the activation of cyclin D1, but prior to the activation of cyclin E which is required for transit through G1 in normal human lymphocytes mitogenically stimulated by phytohemagglutinin (89). In subsequent studies ST ~~mediated reduction in~~ pRb phosphorylation and CDK2 activity ~~was shown to be associated with~~ increased levels of p27 ~~which was found~~ bound to CDK2 (90). The involvement of pRb in ST mediated arrest was examined in the human bladder carcinoma cell line 5637 which lacks a functional pRb and is not arrested in G1 by ST. Once these cells are transfected with a functional pRb they can be arrested in G1. The ST treated pRb transfected cells illustrated that the G1 arrest is associated with a reduced cyclin E/CDK2 activity and with the induction of both p21 and p27. Furthermore, p21 was found to be associated with the Thr-160 phosphorylated, active, form of CDK2 ~~suggesting~~ that the mechanism of ST mediated G1 arrest in these cells is dependent on a functional pRb protein which is hypophosphorylated due to inhibition of cyclin E/CDK2 activity and association of this complex with p21 (91). There are other protein kinase inhibitors besides ST which also target the cyclin E/CDK2 complex and result in the arrest of cells in G1. One such agent is Flavopiridol, a N-methylpiperidinyl, chlorophenyl flavone which can inhibit cell cycle progression in either G1 or G2 (92). Even though Flavopiridol is a potent inhibitor of both recombinant CDK2 and CDK4 kinases *in vitro*, this agent also arrests pRb+ve and pRb-ve cells with equal potency *in vivo*. Since the major kinase complex required for pRb phosphorylation is cyclin D/CDK4, the flavopiridol mediated arrest in pRb negative cell lines without the regulatory interplay between cyclin D/CDK4/pRb, suggest that cyclin E/CDK2 or cyclin A/CDK2 may be an important antiproliferative target of this agent (93). Olomoucine and Roscovitine, two structurally related purine analogues, represent the most novel class of inhibitors which target CDKs. Olomoucine and Roscovitine arrest cells both at the G1/S and the G2/M boundaries, targeting CDK1 and CDK2 with IC50s in the range of 0.7 μM (Roscovitine) and 7μM (Olomoucine) (94,95). Both compounds also target CDK5/p35 complex with similar efficacy to that of CDK1 and CDK2. Although the target of these inhibitors ~~are~~ the CDKs, *in vitro* screening of 60 human tumor cell lines revealed no correlation between the sensitivity of the cell lines to these agents and the presence of wild-type or mutated p53. These results suggest that cell cycle effects of these novel purine analogues may occur via direct inhibition of cyclin/CDK complexes without up-regulation of p21.

CYCLIN E AND RETINOBLASTOMA

The factors regulating cyclin E expression and activity, the timing of cyclin E expression, complex formation with CDK2, and its associated kinase activity during the normal cell cycle are highly suggestive of its function not only in the initiation of S phase but also in the transcriptional regulation of genes required for cell proliferation. Our knowledge of physiological cellular targets for cyclin E/CDK2 is rather limited. However, one family of proteins whose functionality seems to depend on the cyclin E/CDK2 activity is retinoblastoma (pRb) tumor suppressor proteins. pRb, which appears as a hypophosphorylated protein at the beginning of G1 phase, is sequentially phosphorylated throughout the cell cycle by different cyclin/CDK complexes (96-98). The role of cyclin D/CDK4 or CDK6 complex in the regulation of phosphorylation of pRb in early G1 is already substantiated (99) and an association of cyclin D1 with pRb in the absence of a kinase subunit has been documented (100-102). In addition cyclin D has been found to be

[2]The negative regulatory role of p27 on cyclin E/CDK2 activity also has developmental consequences. For example when CG-4 cells, a bipotential oligodendrocyte precursor glial cell line, were induced to differentiate into astrocytes, increases in the levels of p27 were observed which were accompanied by a block in the the kinase activity of cyclin E-CDK2 complexes (84).

dispensable for the G1 checkpoint control in cells which are RB gene-deficient (103) and phosphorylation of pRb may be the rate-limiting event for the G1/S transition which is controlled by cyclin D1 and not cyclin E (104). Hence, the role of cyclin E/CDK2 as a kinase complex required for late G1/early S phase phosphorylation of pRb is more subtle. In the initial investigations which suggested cyclin E was required for pRb regulation, the RB gene was introduced into SAOS-2 osteosarcoma cells, which lack functional pRb, and prevented cell cycle progression. Constitutive expression of cyclins A and E, but not D1, resulted in the release of pRb-mediated growth arrest presumably through the phosphorylation of pRb. The hyper-phosphorylation of pRb and rescue of pRb blocked cells by ectopic expression of cyclins E and A suggests that these cyclins can act as regulators of pRb function by promoting its phosphorylation (97). In a subsequent study designed to analyse the cell cycle-dependent regulation of pRb, a pRb expression system was established in the yeast *Saccharomyces cerevisiae*. These studies demonstrated that multiple yeast G1 cyclins can not only faithfully hyperphosphorylate mammalian pRb, but ectopic expression of human cyclin E and D in these yeast cells complemented the function of yeast G1 cyclin in the hyperphosphorylation of pRb (96). These results implied that multiple G1 cyclins, including cyclin E, are involved in promoting cell cycle progression via pRb hyperphosphorylation.

Other studies investigating the direct transcriptional repression of pRb and a related protein p107, demonstrated that such repression involves protein-protein interaction and is regulated by phosphorylation. Mutation in pRb allowing for its constitutive hypophosphorylation, potentiated repression, while phosphorylation promoted by cyclin A or E reversed this direct transcriptional repression (105). Cyclin E is also found in a multiprotein complex containing E2F, CDK2 and p107 in the late G1 phase of the cell cycle (106) suggesting that this cyclin may be essential for the onset of E2F transcriptional activity in G1. The cyclin E/E2F complex formation was detected primarily during the G1 phase of the cell cycle and decreased as cells entered S phase, where cyclin A/E2F complexes are predominant. p107 is present in both of these complexes which appear in a temporally regulated manner during the cell cycle suggesting a critical role for cyclin E in the transcriptional regulation of genes which drive the G1 to S transition (106). Other studies investigating the involvement of cyclin E in the regulation of p107, revealed that the phosphorylation of p107, like pRb, is also cell cycle regulated and that although the cyclin D containing complexes play a major role in phosphorylating p107 (107), cyclin E may also contribute to the p107 phosphorylation process (108). Overproduction of cyclin E/CDK2 (and cyclin A/CDK2) complexes lead to an intermediate p107 mobility upshift effect indicative of its role in phosphorylation of p107 (108). In addition over-production of cyclin E kinase reversed a p107 mediated G1 block, suggesting that the cyclin E-binding domain of p107, which acts by sequestering endogenous cyclin E kinase (109), contributes to a p107 mediated growth arrest.

Another pRb related protein, p130, has also been shown to physically interact with both cyclin A and E, but not D1, suggesting that p130, like pRb and p107, can be targeted by cyclins A and E for phosphorylation by cyclin/CDK complexes (110). In fact, p130 was shown to be phosphorylated in kinase assays following immunoprecipitations by anti-cyclin E and A antibodies *in vitro*. The above studies suggest that although the physiological relevance of cyclin E/CDK2 in p130, p107- or pRb-E2F complexes remains unclear, they may function in a growth promoting fashion within the transcriptional regulation of genes required for the G1 to S transition.

CYCLIN E IN EMBRYONIC CELL CYCLE AND DEVELOPMENT

Investigations ~~into~~ of cyclin E ~~and its homologues' role~~ in a variety of ~~other~~ fauna besides *Homo sapiens* such as *Drosophila melanogaster*, *Xenopus laevis*, zebrafish and mice have yielded much information about its role ~~not only~~ in cell proliferation ~~but also in~~ and early development. Its role across genera is strikingly similar and suggests that cyclin E not only promotes cell cycle entry into S-phase but is also involved in processes of invertebrate development, such as endocycling and eye pattern development (111,112).

Cyclin E in Drosophila

Drosophila ~~serves~~ is as an excellent system for cell-cycle investigations ~~due to the ease of analysis of~~ since gene function and ~~control~~ regulation is easily analyzed *in vivo* relative to mammalian systems. In *Drosophila*, oogenesis is meroistic, cytoplasmic connections remain between 16 cytocysts and the oocyte is provided with adequate maternal biochemical machinery for 13 complete zygotic cell cycles (113). Cycles 14-16 are dependent upon zygotic transcription and protein synthesis and are timed at the G_2/M phase transition by regulated expression of *String*, a Cdc-2-activating protein (114-116). There is no G_1 in cycles 1-16. Cells may pause in G_1, depending on the tissue, after cycle 16 or 17 to undergo differentiation (113). It was predicted that a cyclin E like protein may function in progression of cells from cycle 16 to 17 and in the development of a G_1 phase.

Homologues of cyclin E were identified in *Drosophila* with probes complimentary to the cyclin box of human cyclin E during screening of cDNA libraries (117). Hence, *Drosophila* cyclin E was cloned because of its homology to human cyclin E, ~~and because~~ It functions similarly to human cyclin E in that it rescues CLN-deficient yeast mutants and ~~it~~ forms complexes with a *Drosophila* CDK2 homologue (118).

Table 1. Homology of Cyclin E Protein Sequences Across Species

Species	Human	*Drosophila*	*Xenopus*	Zebrafish
Drosophila	43%	100%	----------	42%
Xenopus	59%	----------	100%	55%
Zebrafish	60%	42%	55%	100%

Comparison between *Drosophila*, *Xenopus*, Zebrafish and human cyclin E protein were adapted from references (117,119,121) and presented here as percent homology across species.

The identification of cyclin E in other species was also performed on the assumption of homologies. Human cyclin E cDNA was used to screen cDNA libraries of *Xenopus* (119) and mice (120), while *Drosophila* cDNA probes were used to isolate homologues in zebrafish (121). The screening revealed an extensive homology of cyclin E across species as summarised in Table 1, which ~~is~~ highly suggestive ~~of~~ a similar role ~~of~~ cyclin E in cell proliferation and development.

Cyclin E in S-phase

Several investigations analysed the role of cyclin E homologues in S phase progression in lower eukaryotes. These studies suggested that cyclin E controls/promotes entry into S-phase not only in humans but also in *Drosophila*, *Xenopus*, and zebrafish(18,120-122).

Several studies elucidate the role of cyclin E prior to and during *Drosophila* S-phase. Cyclin E is involved in the pattern of gene expression of Proliferating Cell Nuclear Antigen (PCNA), DNA polymerase α and the two ribonucleotide reductase subunits which are constitutively expressed during the first 16 cycles of *Drosophila* embryogenesis (123). Following the appearance of G_1 in cycle 17, constitutive expression of these four genes is terminated and future expression occurs in an identical, complex and spatiotemporal pattern precisely coincident with the onset of S phase and periodic appearance of cyclin E (123). Heat-shock-induced expression of cyclin E in *Drosophila* embryos resulted in the widespread transcriptional activation of the above proteins characteristically seen prior to S-phase even in tissues that are normally silent for the remainder of embryogenesis following entry into G1 (123). Hence, the programmed expression of the S phase genes in *Drosophila* embryos is developmentally regulated by cyclin E and is analogous to the START program of transcriptional control activated by G1 cyclins in *S. cerevisiae* (124,125).

The role of cyclin E during *Drosophila* embryogenesis and control of S phase progression were substantiated in studies where the ectopic expression of cyclin E was shown to induce entry into S-phase, resulting in the accumulation of the G_2 cyclins, and another complete cell cycle in dorsal epidermis of *Drosophila* embryos at the first gap phase, after cycle 16 (18). Conversely, analysis of cyclin E mutant embryos revealed that lack of functional cyclin E after cell cycle 16 (18), the point at which maternal biochemical supplies ~~are thought to~~ expire (117), results in termination of endoreduplication and embryogenic proliferation since the mutant embryos did not progress through S-phase (18). Thus, cyclin E is required for S-phase progression and cell proliferation and it seems likely that down regulation of cyclin E is necessary for the arrest of proliferation at key points during embryogenesis (18).

Studies in *Xenopus* are consistent with the aforementioned role of cyclin E in S-phase initiation in Drosophila. Cyclin E accumulation in *Xenopus* oocytes, observed with its expression and binding to *Xenopus* CDK2, has been linked to oocytes acquiring the ability to replicate DNA (119). In addition, baculovirus-produced cyclin E-CDK2 complexes promote S-phase entry in CDK affinity-depleted *Xenopus* egg extracts in which protein synthesis is inhibited (122). The authors concluded that cyclin E and cyclin A act as an S-phase promoting factor, SPF, in *Xenopus* . However, cyclin E alone does not have SPF activity, it must be complexed with CDK2 to exert its effects as measured by H1 kinase activity (122). This view is consistent with the notion that cyclin E binds exclusively to CDK2 in *Xenopus* during early embryogenesis (126). Collectively, the studies in *Xenopus* eggs and *Drosophila* suggest that not only does overexpression of cyclin E induce entry into S-phase, but immunodepletion (17) or mutation of cyclin E can prevent entry into S-phase.

Cyclin E and Ancillary Proteins: (E2F, RBF, and Dacapo) in *Drosophila*

Consistent with its role in promoting S-phase entry in *Drosophila*, cyclin E also plays a direct role in the E2F mediated transactivation of genes such as

cyclin E itself, which requires renewed transcription at the G_1-S boundary (123). The cyclin E gene has an E2F binding site in its promoter making the transcription factor necessary for cyclin E activation in mice (120) and *Drosophila* (123), similar to human cyclin E (discussed earlier). Even though ectopic overexpression of cyclin E can bypass the E2F requirement and induce S-phase in *Drosophila* embryo cells exhibiting the G_1 lag phase, overexpression of E2F in transgenic *Drosophila* cannot bypass the loss of cyclin E through mutation and induce DNA replication (123).

Cyclin E and E2F demonstrate mutual induction, but the order of induction varies within different developmental situations and cell types within the *Drosophila* embryo (123). Tissues in *Drosophila* embryogenesis deviate from a single pattern of cell cycle after the third or fourth cycle following appearance of G_2 phase (113). The epidermal and mesodermal cells arrest and begin to differentiate, while neuroblasts continue rapid cycling void of gap phases and cells of the endoderm begin endocycling (113). Cyclin E transcription is dependent upon E2F in differentiating cells exhibiting a G1 phenotype since E2F mutants produce no detectable accumulation of cyclin E mRNA and arrest prior to S phase (123). In addition ectopic overexpression of cyclin E or activation of E2F will trigger S-phase initiation in the cellularised cells of cycles 16 or 17. On the other hand, in tissues that lack G_1, such as *Drosophila* developing CNS cells, which express cyclin E constitutively, cyclin E reverses its relationship with E2F and induces E2F activation, suggesting that these cells have an E2F independent source of cyclin E. Studies in cells with constitutive cyclin E expression suggest that the hierarchical relationship between E2F and cyclin E is reversible and tissue specific; cells with an E2F independent source of cyclin E lack G_1, and cells which constitutively express cyclin E may be under the control of a yet to be identified developmental mechanism (123).

One of the key proteins in the pathway by which cyclin E induces E2F in *Drosophila* is RBF which shares structural homology to the mammalian retinoblastoma protein (pRB), p107, and p130 and can bind stoichiometrically with *Drosophila* E2F in its DNA-binding complex (127). Hypophosphorylated RBF binds to and prevents E2F from inducing gene transcription, while cyclin E/CDK2 homologues phosphorylate RBF in *Drosophila*, thereby activating E2F. Collectively these studies suggest that RBF, in *Drosophila* , and the retinoblastoma proteins in mammals, serve as an intermediary allowing cyclin E induction of E2F (127). This also points to the possibility of a positive feedback loop of cyclin E via RBF and the E2F pathway. Cyclin E could indirectly activate its own transcription by acting in tandem with CDK2 to phosphorylate RBF, which allows E2F to bind the cyclin E promoter and activate transcription.

With the description of the cloning and characterisation of Dacapo, encoding a CKI of the p21 and p27 family, another level of regulation of cyclin E expression in *Drosophila* embryos has been established (128,129). As stated above the pattern of cyclin E expression is constitutively high during mitosis 1-15 of embryogenesis and is downregulated during cycle 16, resulting in G1 arrest. Dacapo, which is expressed for the first time in late cycle 16 close to the time of cyclin E and E2F downregulation, binds to and inhibits cyclin E/CDK2 complexes and is required for cells to enter G1 and stop proliferation. Overexpression of cyclin E or lack of Dacapo expression results in the same phenotype, an extra S phase immediately after mitosis 16. Furthermore, overexpression of Dacapo results in a G1 arrest with similar consequences as a cyclin E mutation. Lastly, the G1 arrest mediated by overexpression of Dacapo is completely rescued by cyclin E overexpression (128). Hence, the generation of the first G1 phase during *Drosophila* embryogenesis is dependent not only on the inhibition of cyclin E expression, downregulation of E2F, but also on the expression of Dacapo (130).

Cyclin E, the endocycle and oocyte determination

Constitutive, rather than periodic, expression of cyclin E and its association with CDK2 in early zebrafish embryogenesis and *Drosophila* embryonic cell cycles 1-16 suggest a role for this cyclin in development in addition to its established role in mediation of cell cycle progression (121,126). Cells in early embryogenesis and oogenesis can undergo endocycles which involve DNA replication without mitosis, thus, increasing the ploidy of cells (112). Cyclin E levels have been shown to oscillate during the endocycle and may be involved in the progression and unique characteristics of the endocycle (112). Trials in endocycling cells of *Drosophila* oogenesis suggest two roles for cyclin E within the endocycle. First, it is required for S-phase and ongoing DNA replication. Second, cyclin E may also inhibit the rereplication of DNA. Low levels of cyclin E are proposed to reset DNA replication machinery prior to complete replication of the genome and turn off the cyclin E down-regulation mechanism. This allows cyclin E levels to rise and, in turn, begins DNA replication anew. The truncated S-phase of the endocycle due to declining cyclin E levels resetting the DNA replication machinery results in reduced levels of late-replicating DNA in endoreduplicating cells. Incomplete replication suggests that the checkpoint linking unreplicated DNA to the cyclin E oscillator must be lacking. Cyclin E mutants exhibiting partial loss-of-function exhibit increased amounts

of late-replicating DNA content and less dramatic cyclin E oscillations than the wild type in endocycling cells (112). Increasing levels of cyclin E are proposed to eventually reactivate the down regulation machinery propagating this unique cell cycle variation.

Cyclin E in Development

Cyclin E is an established target of regulatory mechanisms that coordinate not only cell proliferation but also certain developmental events, exemplified in *Drosophila* eye pattern formation (105), where the future eye cells pause in G_1 to differentiate and then reenter the cell cycle. Ectopic expression of cyclin E via heat shock induction at the onset of this proliferative hiatus induces eye cells to complete one entire cell cycle. The premature entry into S-phase caused by ectopic overexpression of cyclin E disrupts eye pattern development in *Drosophila* , illustrating the importance of cyclin E transcriptional regulation in the coordination of proliferation and differentiation (111).

Cyclin E also influences developmental events or outcomes prior to fertilisation. The fate of nurse cells and the oocyte in *Drosophila* oogenesis may be determined by cell-cycle programming (112). Recall that oogenesis is meroistic in *Drosophila* ; two of the sixteen cystocytes have four ring canals and only one of these is the oocyte proper. Germ-line cysts from the ovaries of females mutant for cyclin E often produce two, or even three, oocytes rather than the lone oocyte produced in the wild-type (112). This is a significant finding because other mutations generally result in 16 nurse cells without an oocyte. There are two possible routes by which normal cyclin E may influence oocyte development. First, cyclin E mutations may alter the cell-cycle state of cells immediately following the last cystocyte division. The pro-oocyte is the only cystocyte to enter the meiotic pathway after cystocyte division is complete in wild-type germ-lines. A cyclin E mutation altering the cell-cycle state of other cystocytes may promote their entry into the meiotic pathway (112). Wild-type cyclin E may affect the future nurse cells' cell cycle state after cystocyte division, thereby preventing them from becoming meiotic. Secondly, mutant cyclin E may increase the sensitivity of cystocytes to oocyte determinants which are transported from the future nurse cells to the pro-oocyte (112). Most ectopic oocytes arise from the second cystocyte with four ring canals which normally results in a nurse cell. This may be due to the fact that it is the second cystocyte with four ring canals that is exposed to the most oocyte determining factors while they are in transit to the pro-oocyte. Whatever the mechanism, normal cyclin E function appears to prevent aberrant oocyte development in *Drosophila* oogenesis.

How is cyclin E regulated in lower eukaryotes?

In each organism considered, there is a point at which cyclin E levels oscillate due to maternal-zygotic transition, post-translational modification, negative feedback mechanism and regulation (i.e. Dacapo expression), degradation, transcriptional inhibition via E2F or other mechanisms. A developmental timer, independent of cell cycle progression, has been suggested in the degradation of cyclin E at the midblastula transition in *Xenopus* as another possible mode of regulation (131). Activation of cyclin E protein turnover occurs independently of cell cycle progression in *Xenopus* and does not require protein synthesis. A developmental timing mechanism has been proposed which allows activation of a cyclin degradation pathway after the midblastula transition. The timing mechanism may be held inactive in the eggs until after the zygote's midblastula transition by a mitogen-activated protein kinase signal transduction pathway (131). In addition to offering support for a developmentally timed cyclin E degradation mechanism these results also indicate that cyclin E is stable during early S and M phases of *Xenopus* embryogenesis. Hence, there may be a cell cycle-independent regulator of cyclin E in lower eukaryotes which might accommodate the many roles and interactions cyclin E has in the cell cycle and development.

Cyclin E expression and down regulation have also been demonstrated to be developmentally controlled and independent of progression through the cell cycle in *Drosophila* early embryogenesis (18). In one corroborating study, a *string* mutation was introduced in *Drosophila*, blocking of the cell cycle, to examine if the transcriptional program preceding DNA replication was cell cycle induced (123). Cyclin E transcription would halt in the *string* mutants if it is cell cycle controlled. However, cyclin E transcription continued in a manner consistent with the S phase pattern of wild-type embryos even though the cell cycle had been arrested, suggesting that some developmental factor induced the transcriptional program, including cyclin E, preceding DNA replication independent of cell cycle progression (123).

CYCLIN E AND CANCER

The above discussion emphasises the crucial role of cyclin E in cell proliferation, specifically the transition of cells from G1 to S phase ~~not only~~ in human ~~but in~~ invertebrates and lower eukaryotes, its role in senescence, development, ~~as well as~~ and modulation of downstream signals involving tumor suppressor genes such as pRb. Due to these functional roles of cyclin E it could be inferred that

any defects not only in the maintenance of genetic integrity of this gene but also defects in signals that would modulate the expression and activity of this protein would have profound effects on the regulation of cell proliferation, establishing cyclin E as a prime target for oncogenesis. The remainder of this chapter will discuss the current evidence supporting the role of cyclin E as an oncogene and suggest that the deregulation of this protein is one of the primary events which alter the regulation of checkpoint controls in normal cells, leading to a tumor phenotype.

Different forms of cyclin E

The molecular cloning of cyclin E gene aided in mapping its chromosomal localisation to 19q13.1 in humans and (132-134) and chromosome 7 (i.e. D7Rp2) in mice (135). Although only a few cases of cyclin E gene amplification have been reported (136), there have been several reports on the deregulation of this protein in different forms of cancer (discussed below). However, it is curious that despite a lack of genetic alteration of cyclin E there are several different forms of cyclin E mRNA, presumably resulting from alternative splicing, that exist in proliferating cells. The different forms of cyclin E are presented in Figure 1. In the first report on the alternative splicing of cyclin E, investigators identified a splice variant of cyclin E, termed cyclin Es (137). Cyclin Es lacks 49 amino acids within the cyclin box domain resulting in the appearance of a 43KDa splice variant of human cyclin E compared to the wildtype (WT) 48 KDa (Figure 1). However, this smaller form of cyclin E is unable to associate with CDK2, is inactive in histone Hl kinase assays, and is unable to rescue a triple CLN mutation of *S. cerevisiae* (137). Furthermore, cyclin Es is 1/10th as abundant as wildtype full length cyclin E in several cell lines analysed. Subsequently, in an attempt to determine the presence of any potential alterations in the cyclin E gene in MDA-MB-157, a breast cancer cell line amplifying the cyclin E gene (136), we amplified the entire cyclin E coding region of this cell line by reverse transcription-polymerase chain reaction amplification (RT-PCR), cloned these products and analysed their DNA sequence (138). The PCR products contained two deletional variants of cyclin E termed cyclin E-Δ9 and cyclin E-Δ148 (Figure 1 and (138)). The deletion in clone cyclin E-Δ9 is a 9 base pair in-frame deletion of nucleotides 67-75 at the 5' end of the gene, while the deletion in clone cyclin E-Δ148 is a 148 base pair deletion of nucleotides 1000-1147 at the 3' end of the gene resulting in a frame shift transcript. In order to determine whether the multiple forms of the cyclin E protein detected in tumor cells originated from different transcripts of cyclin E RNA, we performed RT-PCR and found that these two different deletional variants of cyclin E are expressed not only in the MDA-MB-157 tumor cell line, but also in several other tumor and normal cell lines and tissues. In fact, these analyses revealed that the most intriguing feature of the Δ9 and Δ148 variant forms of cyclin E is the lack of distinct differences in their mRNA expression in normal versus tumor cells or tissue samples. Like cyclin Es (137), there is a strong possibility that both cyclin EΔ9 and cyclin EΔ148 identified by our laboratory, are results of alternative splicing since we find potential splice donor and acceptor sites at the deleted junctions of each transcript. However, the cyclin Es variant differs from those we reported in that cyclin Es lacks 49 amino acids within the cyclin box, and is 90% less abundant than the wildtype cyclin E sequence. Unlike cyclin Es, neither the cyclin E Δ9 nor the Δ148 transcripts disrupts the cyclin box, the consensus region which confers activity by its association to a CDK (106). As a result both Δ9 and Δ148 variants of cyclin E retain the ability to functionally bind to CDK2 and phosphorylate histone H1 in insect cells (138). The ability of these novel variants of cyclin E to form an active complex with CDK2 has implications for their biological functions. The Δ148 variant has another interesting feature in that the PEST sequence important for its degradation has been disrupted by this 148 base pair deletion. In fact, the deletion of a PEST sequence has a profound effect on the turnover of cyclin E as its deletion can disrupt ubiquitin mediated degradation of this protein which is enhanced by the auto-phosphorylation of Thr-380 (36,37), present in the aforementioned deleted PEST sequence. Finally, yet another alternatively spliced form of cyclin E has been recently reported which is termed cyclin E-L and contains a 47-bp insertion at nucleotide 140 encoding a protein that is 15 amino acids longer at its amino terminus than the wildtype protein (Figure 1 and (12)). Cyclin E-L has similar functional properties compared to the wild type cyclin E in its binding and activation of CDK2, nuclear localisation and shortening of G1 upon overexpression in cells (12). Although it is not clear why several different alternatively spliced forms of cyclin E are present in human cells, the mere expression of these forms is highly suggestive of very complex transcriptional regulation of this gene. Furthermore, the functional importance of the lower molecular weight protein products of these cyclin E mRNA variants may provide important clues in the deregulation of this cyclin in cancer. After all, it is the lower molecular weight protein products of cyclin E which are expressed and abundant in tumor but not normal cells or tissues (136,139).

Cyclin E as a prognostic marker in cancer

The linkage between oncogenesis and cyclin E was recently reinforced by correlating the deranged expression of cyclin E to the loss of growth control in

expression of cyclin E in tumor cells results in sequestering CDK2 away from other cyclins, such as cyclin A, suggesting that, there is a cyclin E/CDK2 complex which is abundantly and uniformly active in the tumor, but not the normal, cell cycle. One consequence of constitutive activation of cyclin E throughout the tumor cell cycle is the possible phosphorylation of substrates at altered points in the cell cycle resulting in loss of checkpoint control during the progression of G1 to S in tumor cells. We provided evidence for such redundant behaviour of cyclin E/CDK2 in tumor cells by the ability of this complex to phosphorylate pRb under conditions where cyclin D/CDK complexes have been rendered inactive by overexpression of p16 (157). We show that in some breast tumor cells and tissues, the expression of p16 and pRb are not mutually exclusive as has been shown to be the case in other systems (158-162). Overexpression of p16 in cells results in sequestering of CDK4 and CDK6, rendering cyclin D1/CDK complexes inactive. However, pRb appears to be phosphorylated throughout the cell cycle following an initial lag. This time course of *in vivo* pRb phosphorylation is similar to that of *in vitro* GST-Rb phosphorylation achieved by cyclin E immunoprecipitates prepared from these synchronised cells. Hence, cyclin E kinase complexes can function redundantly and replace the loss of cyclin D-dependent kinase complexes which functionally inactivate pRb. ~~Also,~~ The constitutively overexpressed cyclin E is also the predominant cyclin found in p107/E2F complexes throughout the tumor, but not the normal, cell cycle. These observations suggest that overexpression of cyclin E in tumor cells which also overexpress p16 can bypass the cyclin D/CDK4-CDK6/p16/pRb feedback loop, thereby providing yet another mechanism by which tumors can gain a growth advantage (157). The link between cyclin E alteration and pRb inactivation has also been corroborated by two independent studies (29,163). In one study examining the prognostic potential of cyclin E in breast cancer, investigators found that 40% of breast cancer specimens overexpressing cyclin E also showed a defect in pRb (163). In an *in vitro* study examining the role of RB in regulation of cyclin E, fibroblasts from RB gene-negative mouse embryos expressed not only much higher levels of cyclin E protein, but cyclin E's appearance in the cell cycle preceded that of the wildtype cells by 6 hours. Hence, the selective growth advantage that is conferred to the RB-/- cells may be due in part to the deregulation of cyclin E in these cells (29).

The oncogenic potential of human cyclin E has recently been examined in transgenic mice under the control of the bovine ß-lactoglobulin promoter, which directs transgene expression to the mammary glands during pregnancy and lactation (164). This analysis revealed a corroborating role for cyclin E in mammary tumorigenesis as lactating mammary glands of the transgenic mice contained hyperplasia and over 10% of female transgenic mice also developed mammary carcinomas up to 13 months later. Cyclin E may have contributed to the development of these mammary tumors since a transgene specific cyclin E RNA, protein and associated kinase activity were present in the tumor samples obtained from the transgenic mice (164).

SUMMARY AND FUTURE PERSPECTIVE

Human cyclin E was originally identified by its ability to rescue G1 cyclin-defective yeast. It forms a complex solely with CDK2 and is thought to be rate limiting for the G1/S transition during the normal mammalian cell cycle. Figure 4 is a schematic diagram of factors regulating cyclin E expression, activation, and degradation which collectively function to maintain a high enough level of cyclin E to cause progression of cells from G1 to S phase in the normal cell cycle. Some of these factors/pathways have been shown to be altered in cancer cells leading to a consitutively high expression and activity of cyclin E, not just during the G1/S transition but, throughout the tumor cell cycle. Cyclin E deregulation also includes the appearance of multiple isoforms which are present and constitutively active throughout the tumor cell cycle. As a result of such deregulation, substrates may be phosphorylated with an active cyclin E/CDK2 complex at altered points in the cell cycle resulting in loss of checkpoint control during the progression of G1 to S in tumor, but not normal, cells (Fig 4B).

The quantitative and qualitative alteration of cyclin E in tumor cells has been shown to be very useful as a prognostic marker for assessing poor patient outcome. In addition to being a novel prognosticator, cyclin E alteration can also be used as a novel target for cancer therapy. One of the challenges of current chemotherapy is lack of specificity between normal proliferating cells and tumor cells leading to cytotoxicity of bone marrow cells, hair follicles and intestinal epithelium. To eradicate such cytotoxicites that can often lead to life threatening immunosuppression, we require novel targets whose function is to regulate cell proliferation, ~~since it is~~ the deregulation of such targets ~~which~~ gives rise to the transformed phenotype. As a strategy for finding novel targets for cancer therapy which also have causal roles in cell cycle regulation the following agenda could be followed: First, the target of choice should be differentially expressed in normal versus tumor cells. Secondly, once a putative target is chosen which is differentially expressed in normal versus tumor cells, the mechanism of such expression should be elucidated, (i.e., At what level of gene

breast cancer (136,140). We documented several changes in all or most of the breast cancer lines using proliferating normal versus human tumor breast cell lines in culture as a model system. These changes include increased cyclin mRNA stability, resulting in overexpression of mitotic cyclins and cdc2 RNAs and proteins in 9/10 tumor lines, leading to the deranged order of appearance of mitotic cyclins prior to G1 cyclins in synchronised tumor cells. The most striking abnormality in cyclin expression was that of cyclin E. We observed an eight fold amplification of the cyclin E gene in one tumor cell line and aberrant expression in every tumor cell line examined. The deranged production of cyclin E in tumor cells is quantitative and qualitative as cyclin E protein is severely overexpressed in tumor cells and present in lower molecular weight isoforms not observed in normal cells (136). The relevance of cyclin derangement to *in vivo* conditions was directly examined by measuring the expression of cyclin E protein in tumor samples versus normal adjacent tissue obtained from patients with various malignancies (139). These analyses revealed that breast cancers and other solid tumors, as well as malignant lymphocytes from patients with lymphatic leukemia, show severe quantitative and qualitative alteration in cyclin E protein expression independent of the S-phase fraction of the samples. In addition the alteration of cyclin E becomes more severe with increasing breast tumor stage and grade and is more consistent than cell proliferation or other tumor markers such as PCNA, cyclin D1 or c-erb B2. These observations suggested for the first time that the altered expression of cyclin E in the breast tumor samples is not a mere consequence of cell proliferation but represents a significant difference between normal tissue, low and high-stage tumors. These findings support the use of cyclin E as a powerful new prognostic marker for breast cancer (139).

Others have corroborated our findings and demonstrated that immunocytochemical detection of cyclin E identifies tumor proliferation and deregulated cyclin E expression (141). In addition the analyses of archival material of 114 breast tumor specimens from patients with stage I-IV disease for the expression of cyclin E by Western blotting correlated the findings with estrogen receptor status. Furthermore, patients with high cyclin E levels in their tumors had a significantly increased risk of death and/or relapse from breast cancer even if they were node negative (142). In a subsequent study correlating expression of cyclin E and p27 in young breast cancer patients immunohistochemically, a striking stratification of mortality risk was identified when both markers were used (143). The expression of cyclin E and p27 in breast tumors from 278 women aged 20-44 years compared with other tumor characteristics and risk factors revealed that decreasing p27 expression was a significant predictor of poor patient outcome. Furthermore, when a combination of high versus low expression of p27 and cyclin E were used, the prognostic value improved further. In fact, the combination of low cyclin E and high p27 expression correlated with 70% 10 year survivorship and better prognosis, while high cyclin E and low p27 expression was associated with only 20% survival and poor prognosis (143,144). In our own studies (manuscript in preparation) where we examined tumor specimens from 400 breast cancer patients, comparing the changes of cyclin E expression by western blot analysis with seven other established tumor markers, we observe that cyclin E protein is the most consistent marker for determining the prognosis of early-stage node-negative ductal carcinoma. Collectively these studies suggest that cyclin E is a novel prognostic marker for breast cancer (145).

The method of detection of cyclin E in tissue specimens is also critical to assess its role as a prognostic marker. Cyclin E is a nuclear protein (12) and its localisation to the nucleus does not change in normal versus tumor cells (Figure 2). As shown in figure 2, both normal MCF-10A cells, expressing only the 50KDa protein product of cyclin E, and MDA-MB-157, overexpressing the 50 KDa cyclin E as well as the lower molecular weight isoforms of the proteins ranging in size from 33-50KDa, were stained with a cyclin E antibody used for Western blot detection of all the lower molecular weight isoforms of this cyclin. This analysis revealed that there were no distinct differences between normal and tumor cells as to the localisation of the cyclin E signal; in both cases the signal was localised to the nucleus. This was surprising since different molecular weight iso-forms of cyclin E are present in abundance in tumor cells and we hypothesised that some forms would not be localised to the nucleus. Hence, the same antibody which detected the low molecular weight isoforms of cyclin E in immunoblot assays stained only the nucleus of both normal and tumor cells, suggesting that all the lower molecular weight isoforms of cyclin E are localised in the nucleus. The difference between normal and tumor cells was not in localisation, but in signal intensity. Signal intensity in normal cells is much less than that of the tumor cells. In addition, the intensity of nuclear staining in each cell type correlated with the level of cyclin E expression found by immunoblotting. Detection of cyclin E by Western blot analysis is more informative as the antibody will detect any quantitative and/or qualitative alterations associated with cyclin E. This point is presented in Figure 3 where cell extracts from a panel of 10 tumor tissue specimen obtained from breast cancer patients diagnosed with different clinical stages of the disease were subjected to

Western blot analysis with an antibody against cyclin E. As illustrated, cyclin E antibody detected not only the 50 KDa form but also the lower molecular weight isoforms of the protein which are characteristic of the stage of the disease. Immunohistochemical analysis can not differentiate between the lower and higher molecular weight isoforms of cyclin E and as such cannot accurately predict the outcome of the disease.

Amplification and overexpression of cyclin E in cancer

We originally observed an eight fold amplification of cyclin E in only one of 10 breast cancer cell lines examined. Yet, the overexpression of cyclin E was apparent in all 10 cell lines, suggesting that the major mechanisms leading to alteration of cyclin E protein are not a result of amplification of the gene (136). Others corroborated our findings in breast (140) as well as other cancers. For example, cyclin E was found to be amplified in one of 47 colorectal carcinoma cell lines examined in one study (146). In another study, examination of eight human gastric cell lines and 45 human gastric carcinoma tissues revealed that while cyclin E was amplified in only one cell line, a 3-7 fold gene amplification was observed in 7/45 tissue specimen (147). In yet another study, cyclin E gene amplification was detected in 5/53 primary colorectal carcinoma tissues, 3 of which also amplified the CDK2 gene simultaneously with rearrangements (148). Lastly, in a large study examining the involvement of cyclin genes in the genetic alterations of human cancer, a series of 1,171 breast and 237 ovarian tumors were tested for DNA amplification of 5 cyclin genes including cyclins E and D (149). This study revealed that cyclin D1 was amplified in 12.6% of all breast tumors while cyclin E never, or only on rare occasions, showed increased DNA copy numbers. Collectively these studies emphasise that the changes leading to cyclin E deregulation, in at least breast cancer, do not occur at the DNA level and mainly affect expression and regulation of the protein. Several studies have corroborated this hypothesis. For example, when the expression of cyclin E was investigated by Western blotting in human colorectal carcinomas and in non-nepotistic colorectal mucous it was observed that the level of this cyclin was much higher in the cancer tissue than in the non-nepotistic mucous in 92% of the patients (35 out of 38) (150). This being the case, it is believed that cyclin E may be one of the factors contributing to the uncontrolled high-speed division of colorectal cancer cells. Similar results were obtained when cyclin E protein levels were analysed by Western blotting in 19 patients with acute lymphoblastic leukemia (151). Whereas normal, nonproliferating peripheral blood mononuclear cells expressed low levels of the 50-KDa cyclin E protein, the leukemic cells demonstrating low proliferation status expressed not only high levels of the 50 KDa cyclin E protein, but also high levels of the lower molecular weight isoforms of this protein which correlated to the relative malignant status of the cells (151). In yet another study, the expression of cyclin E in human colorectal adenomas and adenocarcinomas revealed that the cyclin E was overexpressed in 25% (91/358) of the adenomas and 56% (149/267) of the adenocarcinomas by immunohistochemical analysis (152). The overexpression of cyclin E was higher in the malignant adenocarcinomas than in the more benign adenomas and significantly correlated with the proliferative activity of tumor cells and as such was suggested to contribute to the development and early progression of the colorectal carcinomas (152). Overexpression of cyclin E was also evident in localised prostate cancer (153) and in most chronic lymphocytic leukemias (154). Overexpression of cyclin E (and D1) was also observed in N-nitrosomethyl-benzylamimine(NMBA)-induced rat esophageal tumorigenesis and was found to increase as the state of tumorigenesis advanced (155). There was a sequential increase in cyclin E-positive cells from normal epithelium, to preneoplastic lesions, to papillomas suggesting that overexpression of cyclin E (and D1) occurs relatively early in rat esophageal tumorigenesis and may participate in such a model of tumor progression (155). Carcinogen induced rat mammary carcinomas also displayed abnormalities in the expression of several cell cycle regulated proteins, including cyclin E, associated with tumor progression (156). Similarly, cyclin E overexpression and appearance of its lower molecular weight isoforms were observed in an *in situ* system composed of hyperplasia, preneoplasia and neoplasia of mouse mammary glands (156). Collectively the above studies examining the expression of cyclin E in tumor cell lines, and tissue specimens suggest that cyclin E overexpression not only plays a major role in the steps involved in tumor progression but that it might also be used prognostically in several different types of cancers, as its overexpression is universal.

Deregulation of cyclin E in cancer

The mechanism of cyclin E alteration is in part a result of its deregulation in breast cancer. The alteration of cyclin E in breast cancer has been further characterised and reveals that while cyclin E is cell cycle regulated in normal cells, it is present constitutively and in an active CDK2 complex in synchronised populations of breast cancer cells (138). Cyclin E is present in altered forms in synchronised populations of tumor cells throughout the cell cycle and the kinase activity associated with it, or with CDK2, is also constitutively active. In addition the abundant and constitutive

regulation is the differential expression of the target gene controlled in normal versus tumor cells.) Lastly, and by far the most challenging step is to exploit therapeutically the mechanism of differential expression of the target gene in tumor cells. The Cyclin E/CDK2 complex provides us with such a novel target whose inhibition by chemical agents could ~~potentially~~ protect normal cells against the toxic affects of chemotherapeutic agents by arresting them in the G1 phase of the cell cycle. Tumor cells on the other hand, will not arrest in G1 since they lack the checkpoint controls which regulate their progression to S phase. Once the normal cells have been protected by inhibition of cyclin E/CDK2 activity, the subsequent administration of standard chemotherapeutic agent would only target the proliferating tumor cells, not the arrested normal cells. Such a treatment strategy could result in the eradication of the tumor without toxicity to the host.

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Figure legends:

Figure 1: Alternatively spliced forms of cyclin E. Cyclin E WT: Cyclin E wild-type; Cyclin E Δ9 and Δ148 were adapted from reference (138); cyclin ES adapted from reference (137); and cyclin EL adapted from reference (12).

Figure 2: Nuclear localization of cyclin E in normal and tumor human breast cancer cell lines. Asynchronous (A and B) tumor MDA-MB-157 and (C and D) normal immortalized MCF-10A cell lines were cultured on glass coverslips, fixed and permeablized with 3.7% formaldehyde and triton X-100. They were then washed, stained with anti-cyclin E monoclonal antibody (HE12), washed and incubated with Texas red-conjugated goat anti-mouse immunoglobulin G antibodies. After washing, coverslips were mounted with an antifader.(B and D) are phase contrast images of the same field. Fluorescence (1 min exposure time for each cell line) and phase contrast (1 sec. exposure time for each cell line) images were obtained with Nikon FX-35A attached to an Optiphot Nikon microscope using a 100X objective.

Figure 3: Abnormal expression of cyclin E protein in human breast tumor tissue. Whole cell lysates were extracted form 10 breast cancer tissues as well as 76N normal and MDA-MB-157 tumor cell lines. Breast cancer types and tumor stages are: Lane 1, Stage 2 Ductal Carcinom In Situ (DCIS); Lane 2, Stage 1 DCIS; Lane 3, Stage 1 DCIS, Lane 4, Stage 4 Invasive ductal carcinoma (metastasis to Omentum); Lane 5, Stage 4 Invasive ductal carcinoma (metastasis to Omentum); Lane 6, Stage 2 DCIS; Lane 7, Stage 1 DCIS; Lane 8, Stage 4 Invasive ductal carcinoma (metastasis to ovaries); Lane 9, Stage 3 Invastive ductal carcinoma (no metastasis); Lane 10, Stage 2 DCIS. Protein extracts were analyzed on Western blots (50 µg protein/extract/lane) and hybridized with affinity purified anti-cyclin E monoclonal antibody. Molecular weight standards were used to estimate the position of each band.

Figure 4: Cyclin E/CDK2 checkpoint in normal versus tumor cells. pRb: Retinoblastoma protein; pRB-P: hyperphosphorylated pRb; TGFß1: Transfroming growth factor-beta 1; E2F: transcription factor;  : activating effect or promotes;  : inhibitory effect or inhibits;  : inhibitory effect not present or inactive; ????: step or mechanism unknown.

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Advances in Brief

Lovastatin Induction of Cyclin-dependent Kinase Inhibitors in Human Breast Cells Occurs in a Cell Cycle-independent Fashion[1]

Julie Gray-Bablin, Sharmila Rao, and Khandan Keyomarsi[2]

Division of Molecular Medicine, Laboratory of Diagnostic Oncology, Wadsworth Center, Albany, New York 12201

Abstract

Cyclin-dependent kinase inhibitors (CKIs) p21, p27, p16, and p15 are an essential and integral part of cell cycle regulation. Studies on the expression of these inhibitors in normal *versus* tumor human breast cancer cells revealed that although p27 and p16 are expressed at higher levels in tumor cells, p21 and p15 expression were higher in normal cells. Analysis on the expression pattern of these proteins throughout the cell cycle in synchronized cells demonstrated a substantial increase in p21 during the S-phase in normal cells and barely detectable expression of p21 in any phase of the tumor cell cycle. Levels of p15, p16, and p27 remained relatively constant throughout the cell cycle of normal and tumor cells. Synchronization of tumor cells by lovastatin, which arrests cells in G_1, resulted in increased levels of p21 and p27 with a concomitant decrease in cyclin-dependent kinase 2-associated kinase activity. Synchronization of cells by double-thymidine block did not result in the induction of p21 or p27. These observations suggest that lovastatin causes a profound cell cycle-independent alteration of CKI expression which is distinct from growth factor deprivation or thymidine block.

Introduction

Cell proliferation, the ability of cells to traverse the cell cycle, is intricately regulated in normal cells by the coordinate activity of both positive and negative regulating proteins. However, tumor cells have defective cell cycle control mechanisms, resulting in the uncontrolled growth and proliferation characteristic of all cancers. The cell cycle is driven forward by complexes of stable kinases termed cdks[3] and unstable regulatory subunits called cyclins (1–4). An additional layer of cell cycle regulation has emerged with the discoveries of low molecular weight CKIs which represent a novel mode of negative regulation (5–7). There are two classes of CKIs: the CIP/KIP family including $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ and the INK4 family including $p15^{INK4B}$ and $p16^{INK4A}$ reviewed elsewhere (8). Both classes of inhibitors function to block the activity of cdks. p21 and p27 inhibit cdk2–5 and cdk6, whereas p15 and p16 inhibit cdk4 and cdk6 (8). CKI levels have recently been examined during the cell cycle by synchronization of cells with the drug lovastatin (9, 10), which is routinely used to treat hypercholesteremia. Lovastatin inhibits the activity of the 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase enzyme, critical to the cholesterol biosynthetic pathway. Among other effects, inhibition of this pathway disrupts protein prenylation and, therefore, also disrupts the subcellular localization and function of proteins such as ras and protein glycosylation via inhibition of dolichol production. As a result the pleiotropic cellular effects of lovastatin are not thoroughly understood or characterized. Lovastatin has been used recently to arrest diverse cell types in both mitotic (11–13) and meiotic cell cycles (14). Hengst *et al.* (9) reported on the induction of p27 in cells synchronized via lovastatin treatment. More recently, it was demonstrated that this induction, rather than being transcriptionally mediated, is at least partially attributable to translational control of p27 (10). The induction of p27 by lovastatin was assumed to be a cell cycle effect rather than a drug-specific effect. Here, we report on the cell cycle expression of four CKIs of both the p21 and p16 families and demonstrate that their expression is modulated by lovastatin via a cell cycle-independent mechanism. Synchronization of human breast cells by means other than lovastatin treatment did not lead to the induction of any of the CKIs examined.

Materials and Methods

Materials, Cell Lines, and Culture Conditions. [*methyl*-^{3}H]thymidine (81 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Lovastatin was kindly provided by A. W. Alberts (Merck, Sharp and Dohme Research Pharmaceuticals, Rahway, NJ). Mevalonic acid lactone and serum were purchased from Sigma Chemical Co. (St. Louis, MO) and cell culture medium from Life Technologies, Inc. (Grand Island, NY). All other chemicals used were of reagent grade. The scintillation cocktail used was Budget-Solve from Research Products International (Mount Prospect, IL). Before addition to cultures, lovastatin and mevalonic acid were converted from their inactive lactone prodrug form to their active dihydroxy-open acid as described previously (12). The culture conditions for 70N, 81N, and 76N normal cell strains, MCF-10A cell line, and MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-436, T47D, BT-20, HBL100, Hs578T, SKBR3, and ZR75T tumor cell lines were described previously (15, 16). All cells were cultured and treated at 37°C in a humidified incubator containing 6.5% CO_2 and maintained free of *Mycoplasma* as determined by the MycoTect kit (Life Technologies, Inc.).

Synchronization and Flow Cytometry. Synchronization by lovastatin treatment or growth factor deprivation was performed as described previously (12). Briefly, medium was removed 36–48 h after the initial plating of MDA-MB-157 cells and replaced with fresh medium plus 40 μM lovastatin for 36 h. At time 0 h, cells were stimulated by replacing the medium with fresh medium containing 4 mM mevalonic acid. Cells were harvested at the indicated times, and DNA synthesis and cell density were measured as described previously (12).

The synchronization of normal mammary epithelial 76N cells by growth factor deprivation is as follows: At 48 h following plating subconfluent 76N cells, medium was removed and cells were washed three times and incubated in DFCI-3 medium for 72 h. DFCI-3 medium is DFCI-1 medium without essential growth factors (17). At time 0 h, cells were stimulated by the addition of DFCI-1 medium and harvested at the indicated times thereafter, and DNA synthesis rates were measured as described previously (12).

76N normal mammary epithelial cell strain and MDA-MB-157 tumor cell line were also synchronized at the G_1-S boundary using a modification of the double-thymidine block procedure (18) as described previously (16). For flow cytometry studies, 10^6 cells were centrifuged at 1000 × *g* for 5 min, fixed with ice-cold 70% ethanol (30 min at 4°C), and washed with PBS (19). Cells were suspended in 5 ml of PBS containing 10 μg/ml RNase, incubated at 37°C for 30 min, washed once with PBS, and resuspended in 1 ml of 69 μM propidium

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[2] To whom requests for reprints should be addressed, at Wadsworth Center, Empire State Plaza, P.O. Box 509, Albany, NY 12201. Phone: (518) 486-5799; Fax: (518) 486-5798; E-mail: keyomars@wadsworth.org.

[3] The abbreviations used are: cdk, cyclin-dependent kinase; CKI, cdk inhibitor; HMG, 3-hydroxy-3-methylglutaryl.

iodide in 38 mM sodium citrate. Cells were then incubated at room temperature in the dark for 30 min and filtered through a 75-mm Nitex mesh. DNA content was measured on a FACScan flow cytometer system (Becton Dickinson, San Jose, CA), and data were analyzed using the CELLFIT software system (Becton Dickinson).

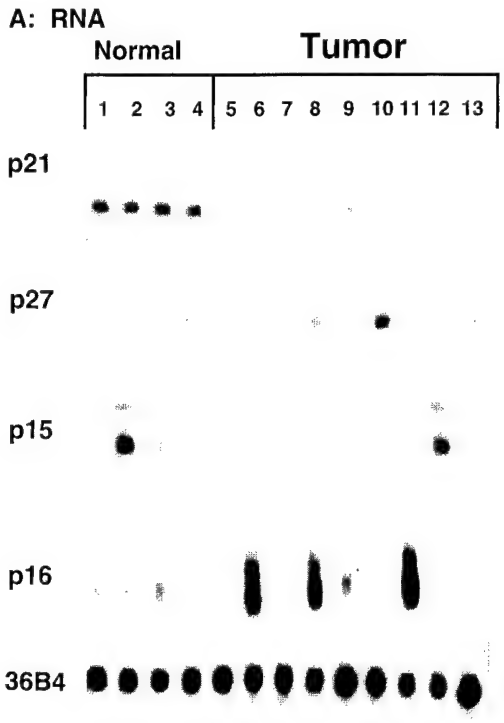
RNA Isolation and Northern Blot Hybridization. Total cellular RNA was extracted from normal and tumor cells by guanidinium isothiocyanate and subjected to cesium chloride gradient purification. For Northern blot analysis, 20 μg of total RNA were fractionated under denaturing conditions on a 1.2% agarose/0.66 M formaldehyde gel and transferred to a Nytran filter (Schleicher & Schuell, Keene, NH) for subsequent hybridization. The DNA probes were prepared by random primed labeling (Boehringer Mannheim, Indianapolis, IN). Vector containing p21 was provided by S. Elledge and W. Harper, cDNA to p27 was provided by Joan Massague, cDNAs to p15 and p16 were provided by David Beach, and cDNAs to histone H4 and 36B4 as described previously (15). All cDNA inserts were labeled with [α-^{32}P]dCTP to a specific activity of 1×10^9 dpm/μg DNA.

Western Blot and Immune Complex Kinase Analysis. Cell lysates and tissue homogenates were prepared and subjected to Western blot analysis as described previously (15, 20). Briefly, 50 μg of protein from each tissue sample or cell line were electrophoresed in each lane of either a 10% SDS-polyacrylamide gel (cyclin A), a 13% SDS-polyacrylamide gel (p21 and p27), a 15% SDS-polyacrylamide gel (p15 and p16), and transferred to Immobilon P. Primary antibodies used were pRb monoclonal antibody (PharMingen, San Diego, CA) at a dilution of 1:100, monoclonal antibody to p16 (a gift from Jim DeCaprio, Dana-Farber Cancer Institute, Boston, MA) at a dilution of 1:20, p15, p27, and p21 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100, affinity-purified rabbit anti-human cyclin A antibody (a gift from J. W. Harper, Baylor College of Medicine, Houston, TX) at a dilution of 1:20,000, and monoclonal antibody to actin (Boehringer Mannheim) at 0.63 μg/ml Blotto.

Immunoprecipitations and H1 kinase assays were performed as described previously (16, 21). For immunoprecipitation followed by Western blot analysis, 250 μg of protein were used per immunoprecipitation with a polyclonal antibody to cdk2 in lysis buffer as described above. The Immunoprecipitates were then electrophoresed on a 13% SDS-polyacrylamide gel, transferred to Immobolin P, blocked, and incubated with either monoclonal antibody to p21 (Oncogene Science, Cambridge, MA) or p27 (Transduction Laboratories, Lexington, KY) at a dilution of 1:100 and analyzed as described above.

Results and Discussion

CKIs Are Differentially Expressed in Normal *versus* Tumor-derived Exponentially Growing Cells. To determine the relative levels of CKIs in normal *versus* tumor-derived breast epithelial cells, we initiated our studies by investigating the expression of four CKIs (p21, p27, p15, and p16) in three normal cell strains, one immortalized cell line, and nine mammary epithelial tumor cell lines (Fig. 1). The three normal cell strains (70N, 81N, and 76N) were established from reduction mammoplasties obtained from three different individuals (17). The immortalized MCF-10A cell line is a subline of a normal breast epithelial cell strain, MCF-10, derived from human fibrocystic mammary tissue which was immortalized after extended cultivation in medium containing low concentrations of calcium (22). The mammary epithelial cell types, estrogen receptor, p53, pRb (retinoblastoma), and cyclin E status of these normal and the nine established tumor cell lines used were described previously (15, 23). Total RNA and protein were prepared from exponentially growing cells and subjected to Northern or Western blot analyses, respectively. Loading controls were performed for both Northern and Western blot analysis using an invariant mRNA (*i.e.*, 36B4 cDNA) and protein (*i.e.*, actin antibody) as probes. As demonstrated, p21 mRNA is expressed at higher levels in normal *versus* tumor cells, whereas p27 and p16 mRNAs are more highly expressed in some tumor cells (Fig. 1*A*). The pattern of p21 expression is interestingly different at the protein level with some tumor cell lines as demonstrated by Western blot analysis (Fig. 1*B*). For example, MCF-7, T47D, BT-20T, and ZR75T cells, all



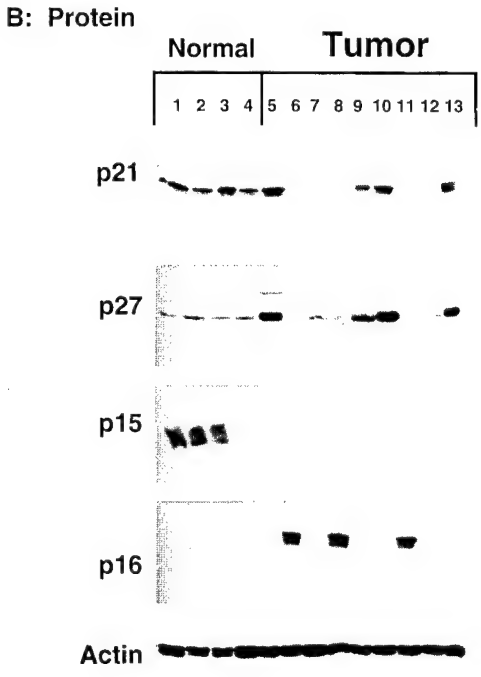


Fig. 1. Altered expression of CKIs in exponentially growing normal *versus* tumor cells. Northern blot (*A*) and Western blot (*B*) analyses of CKI expression in normal *versus* tumor breast epithelial cells. RNA was analyzed on Northern blots (20 μg of RNA/lane). The list of normal cells (*Lanes 1–4*) and tumor cell lines (*Lanes 5–13*) is presented below. Blots were hybridized with the indicated probes or 36B4 (15) used for equal loading. *B*, Western blot analysis of CKIs from cell extracts obtained from the same cell lines used in *A*. Fifty μg of total cell extract were run on SDS-polyacrylamide gels. Proteins were transferred to Immobilon P and blots were incubated with the indicated anti-CKI antiserum, and immunoreactive proteins were detected with the enhanced chemiluminescence reagent (Amersham). Lane assignments: 1-70N; 2-81N; 3-76N; 4-MCF-10A; 5-MCF-7; 6-MDA-MB-157; 7-MDA-MB-231; 8-MDA-MB-436; 9-T47D; 10-BT-20; 11-HBL-100; 12-HS578T; and 13-2R75T. Blots were hybridized with the indicated probes or actin used for equal loading.

estrogen-receptor positive cell lines, express relatively high levels of p21 protein, whereas the levels of p21 mRNA in these cells were very low. In addition, in all of the tumor cell lines that show increased p27 protein levels, the p21 levels are not as low as is typical in tumor cells, but instead are similar in levels to those of normal cells. Although the levels of mRNA for each of the CKIs generally correspond to their protein levels, some particular discrepancies are noted with respect to p15 expression. In the normal cell strains, p15 protein is expressed at high levels, whereas mRNA levels are only increased in the 81N cells (Fig. 1, *Lane 2*). Additionally, the tumor cell line Hs-578T (Fig. 1, *Lane 12*) expresses very high levels of p15 mRNA and undetectable levels of p15 protein. These observations suggest that p15 levels may be regulated by translational or posttranslational mechanisms.

Although the differences in CKI expression observed between normal breast cells and breast cancer cells could be a result of tissue specificity, it is more likely due to changes which occur during tumorigenesis. In most cases where a difference in expression was noted between normal *versus* tumor cells, all four normal cells (*i.e.*, p21) or all three normal cell strains (*i.e.*, p15) consistently exhibited a clearly detectable expression of these two proteins compared to tumor cells, even though the normal cells were derived from different individuals.

Expression of CKIs in Synchronized Normal and Tumor Cells. The CKIs have been proposed to establish a threshold of inhibition that must be exceeded if cell cycle progression is to occur (24). Therefore, a disruption in the levels of CKIs or cyclin-cdks could offset the threshold balance or result in the displacement of particular regulatory proteins. The net result of such an imbalance suggests an aberrant cell cycle progression. This hypothesis led us to analyze the pattern of expression and relative levels of the CKIs, P15, p16, p21, and p27 throughout the cell cycle in synchronized populations of both normal cell strains and tumor cell lines (Figs. 2–4).

Initially, we assessed the effect of growth factors on CKI expression by arresting normal 76N cells in G_0 (Fig. 2; *i.e.*, growth factor deprivation-induced quiescence). This allowed us to determine whether a particular phase of the cell cycle might enrich for the expression of these CKIs. Briefly, cells were cultured in growth factor-deficient medium for 72 h and then stimulated to reenter the cell cycle with the addition of growth factors. Reentry into the cell cycle and S-phase was monitored by [^{3}H]thymidine incorporation (Fig. 2*B*). At the indicated times after readdition of growth factors, cells were harvested and extracted proteins were analyzed on Western blots with antibodies to p21, p27, p15, p16, and cyclin A (Fig. 2*A*). In normal 76N cells, the pattern of expression of cyclin A protein is consistent with that seen for other normal cell types with levels rising dramatically at the S-phase and disappearing by the end of G_2-M. The pattern of expression of the four CKIs examined following growth factor stimulation indicates that p21 levels increase substantially (10-fold) during the S-phase, whereas p27, p15, and p16 levels remain relatively unchanged throughout the cell cycle (Fig. 2*A*). These observations corroborate our hypothesis that these CKIs may each function differently during the cell cycle, with p21 clearly having a pronounced role during the S-phase of cycling cells and p27 and p15 being important in quiescent cells as recently documented in fibroblasts (25).

Induction of p21 and p27 by Lovastatin in Tumor Cells. Unlike normal cells, it is difficult to growth factor deprive tumor cells or arrest them in G_0 since most tumor cells have lost their growth factor requirement. As such a pharmacological method has to be used to synchronize tumor cells in early G_1. For that purpose we used lovastatin, an inhibitor of the cholesterol biosynthetic pathway, to synchronize MDA-MB-157 breast cancer cells in G_1 (Fig. 3). We have previously reported on the use of lovastatin as an agent to synchronize cells in early G_1 (12, 13). Cells were cultured in lovastatin for 36 h, at which time lovastatin media were removed and replaced with media containing mevalonate (the end product of the cholesterol biosynthetic pathway), which is routinely used to stimulate cells to reenter the cell cycle and advance to the S-phase. Synchrony of tumor cells at various times after release from the lovastatin block was monitored by histone H4 mRNA expression and [^{3}H]thymidine incorporation (Fig. 3, *A* and *E*). Immediately following lovastatin treatment there is inhibition of DNA synthesis followed by a dramatic increase in incorporation of [^{3}H]thymidine as well as histone H4 expression. This increase is indicative of the cells being arrested in G_1.

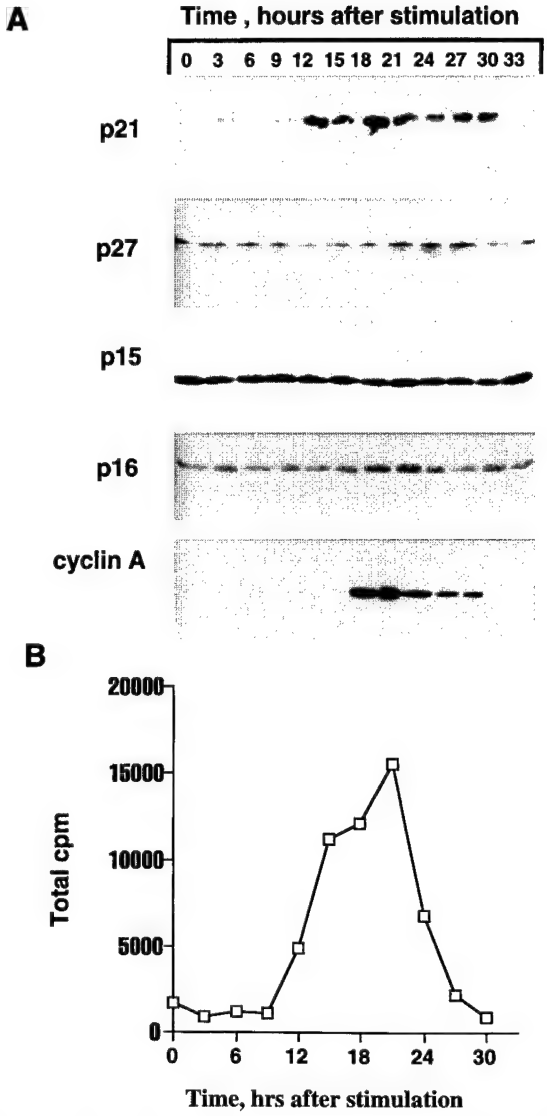


Fig. 2. Expression of CKIs in normal cells synchronized by growth factor deprivation. Normal 76N cells were arrested in G_0 via growth factor deprivation for 72 h and then stimulated to reenter the cell cycle through the addition of growth factors. *A*, at indicated times after growth factor stimulation, whole-cell lysates were prepared and analyzed on Western blots. Blots were incubated with indicated anti-CKI antiserum and immunoreactive proteins visualized with the enhanced chemiluminescence reagent. Detection time: 1–30 s with the exception of p16 for 24 h (see text). Blots were probed with actin used for equal loading (data not shown), and the pattern was identical to that of p15 whose levels do not change during the cell cycle. *B*, DNA synthesis rates as measured by [^{3}H]thymidine incorporation.

Total RNA and protein were extracted from cells harvested at various times after release from lovastatin and analyzed on Northern and Western blots using $p21^{CIP1/WAF1}$, $p27^{KIP1}$, histone H4, and 36B4 cDNAs and p21 and p27 antisera as probes (Fig. 3, *A* and *B*). Unexpectedly, we found that lovastatin treatment dramatically induced the expression of p21 and p27 in MDA-MB-157. These two CKIs are barely detectable in exponentially growing tumor cells (Fig.

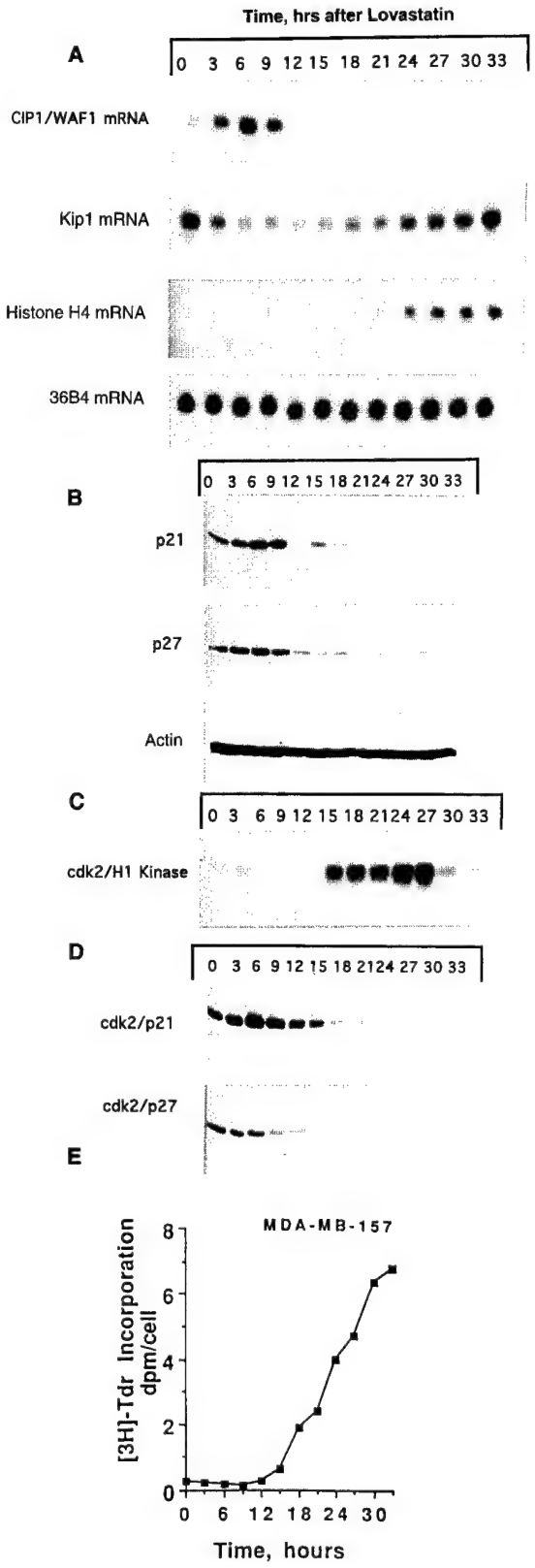


Fig. 3. Induction of the CKIs p21 and p27 by lovastatin is inversely correlated with cdk2 kinase activity. MDA-MB-157 tumor cells were cultured in 20 μM lovastatin for 36 h, at which time lovastatin was replaced by mevalonate. Samples were collected at indicated times after lovastatin removal, and RNA and proteins were extracted. *A*, Northern blot analysis: RNA extracted from cells at indicated times after lovastatin treatment was analyzed by Northern analysis (20 μg/lane) and probed with ^{32}P-labeled p21, p27, histone H4, or 36B4 cDNA. *B*, Western blot analysis: 50 μg of protein extracts from each condition were analyzed using Western analysis with anti-p21, anti-p27, or anti-actin-specific antisera, and blots were developed with the enhanced chemiluminescence reagent. *C*, histone H1 kinase assay: 500 μg of extracts were immunoprecipitated with anti-cdk2 polyclonal antiserum and complexes were assayed for the ability to phosphorylate histone H1. The H1-labeling reaction complexes were analyzed by SDS-PAGE and autoradiography. *D*, immunoprecipitation/Western blot analyses: 500 μg of protein extracts were immunoprecipitated with anti-cdk2 polyclonal antisera and precipitated complexes were analyzed using Western analysis with anti-p21 or anti-p27 monoclonal antisera. *E*, DNA synthesis rates were monitored by [^{3}H]thymidine incorporation.

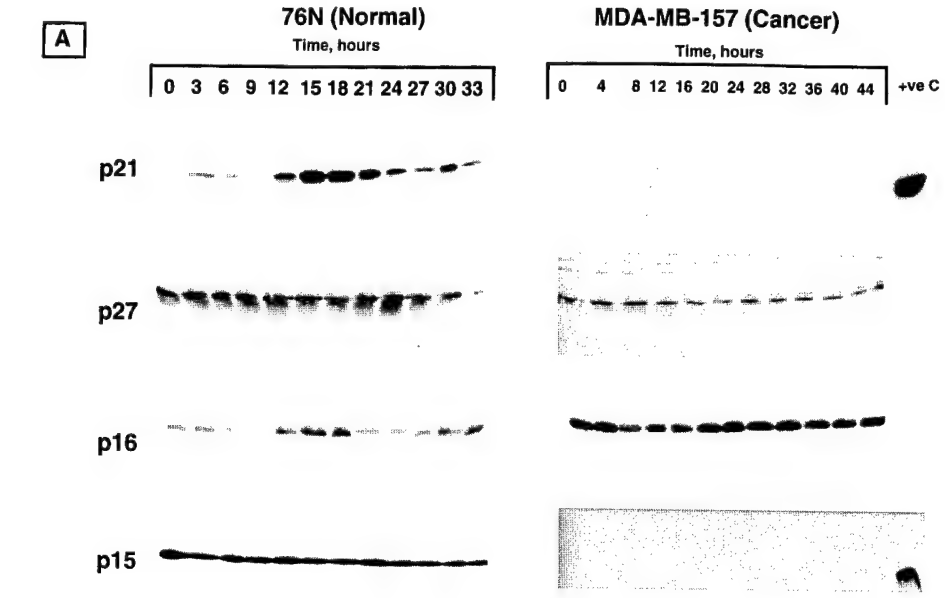
1*B*). The lovastatin induction is evident at both the transcriptional (p21) and translational (p21 and p27) levels as demonstrated by Northern (Fig. 3*A*) and Western blot (Fig. 3*B*) analyses. Although the CKI levels were substantially induced for 9 hours, the levels abruptly diminished. The abrupt induction of p21 and p27 by lovastatin is not due to a response to the medium change since earlier experiments demonstrated that p21 and p27 protein increase during lovastatin treatment (data not shown) and continue until 9 h posttreatment (Fig. 3). To determine whether the transient induction of the CKIs led to the inhibition of kinase activity, we measured the phosphorylation of histone H1 in anti-cdk2 immunoprecipitates prepared from extracts of synchronized cells (Fig. 3*C*). These results indicate that the induced levels of p21 and p27 are accompanied by a dramatic decrease in cdk2-associated kinase activity which then increases upon disappearance of p21 and p27 proteins. To determine whether p21 and/or p27 can directly associate with cdk2 and inhibit its activity, we performed a two-step immunoprecipitation/Western blot analysis. We immunoprecipitated lovastatin-treated cell extracts with anti-cdk2 antisera followed by immunoblotting with p21 or p27 antisera (Fig. 3*D*). Anti-p21 immunoblot analysis of cdk2-containing complexes demonstrates that p21 is directly associated with cdk2 and that the relative amount of p21 associated with cdk2 is inversely correlated with kinase activity of cdk2.

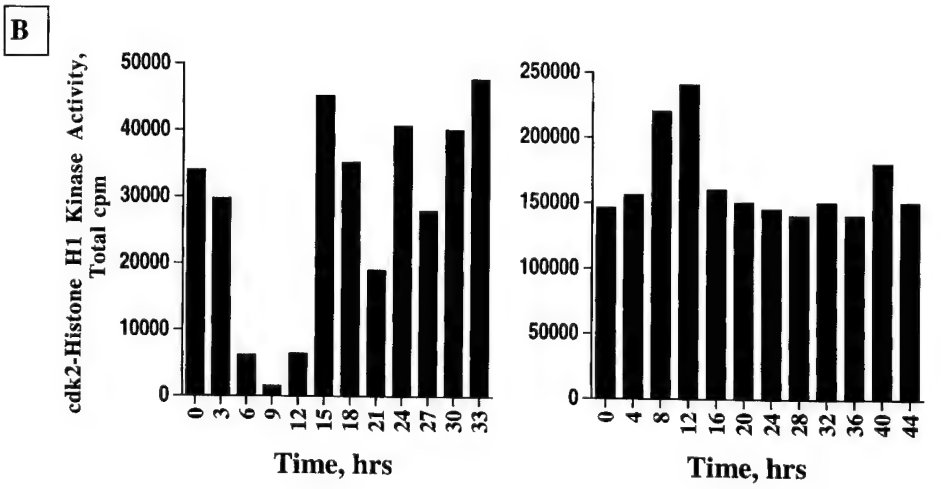
p27 is also similarly associated with cdk2, although in relatively less abundance than p21. Together these analyses indicate that not only are p21 and p27 induced during lovastatin synchronization in MDA-MB-157 tumor cells but they also functionally complex with cdk2. Furthermore, the expression of p21 and p27 is inversely correlated with cdk2 kinase activity, suggesting that these CKIs directly inhibit cdk2.

Induction of p21 and p27 in Tumor Cells by Lovastatin Is Cell Cycle Independent. We synchronized both normal and tumor cell types in the G_1-S-phase boundary by double-thymidine block [(16) and Fig. 4] to determine whether the lovastatin-mediated induction of p21 and p27 is cell cycle dependent or due to a direct (as of yet unknown) effect of lovastatin. This synchronization trial was also used to compare the cell cycle pattern of expression of the four CKIs in normal and tumor cells. Synchrony of both cell types after release from block was monitored by flow cytometry [(16) and Fig. 4*C*]. Cells were harvested at various times after release from treatment, and extracted proteins were analyzed on Western blots with antisera to p21, p27, p16, p15 (Fig. 4), and cyclin A (16). In both normal and tumor cells, the expression pattern of cyclin A protein is consistent with that seen for other cell types, the levels are tightly regulated such that peak expression occurs during the S-phase and early M as documented previously (16).

The pattern of expression of p21 in normal cells revealed cell cycle regulation consistent with the growth factor deprivation results shown in Fig. 2. In normal cells, with both methods of synchronization, p21 is cell cycle regulated with peak levels coinciding with the peak S-phase and early M (Fig. 4*A*, *left panels*). In tumor cells, however, p21 levels remain low and virtually undetectable during all phases of the cell cycle. Thus, we were not able to enrich for p21 expression at G_1 of tumor cells synchronized by double-thymidine block (Fig. 4*B*, *right panels*). These observations suggest that the induction of p21 during lovastatin synchronization (Fig. 3) is not cell cycle dependent but is rather a lovastatin- or mevalonate-specific effect.

The level of p27 remained unchanged in both normal and tumor





C

Time, hrs	G1	S	G2/M
0	57	71	30
3	55	100	54
6	51	45	90
9	72	48	100
12	100	33	47
15	92	85	40
18	65	100	47
21	62	85	57
24	67	47	69
27	81	38	67
30	100	19	57
33	100	18	62

Time, hrs	G1	S	G2/M
0	76	66	27
4	47	100	30
8	20	63	70
12	26	27	100
16	50	25	55
20	65	21	42
24	80	19	40
28	100	19	36
32	97	19	38
36	80	25	36
40	75	30	25
44	70	40	25

Fig. 4. Expression of CKIs in synchronized normal 76N and tumor MDA-MB-157 breast cells. Both cell lines were synchronized using the double-thymidine block procedure: 76N cells were incubated in 2 mM thymidine for 24 h, washed, and incubated in regular medium for 12 h and incubated in 2 mM thymidine for an additional 24 h. MDA-MB-157 cells were treated similarly, except incubation in thymidine was for 36 h and thymidine-free media for 24 h, as described previously (16). At the indicated times following release from double-thymidine block, cell lysates were prepared and subjected to Western blot (*A*), histone H1 kinase (*B*), and flow cytometry (*C*) analyses. Fifty μg of extracts of cells were subjected to Western blot analysis with antisera directed against the indicated CKI or cyclin A at indicated times after double-thymidine block. Note: p21 and p15 +ve C are from 76N cell extracts and are used as a positive control. Blots were probed with actin used for equal loading (data not shown), and the pattern was identical to that of p15 in normal cells and p16 in tumor cells whose levels do not change during the cell cycle. For histone H1 kinase activity, equal amount of proteins (500 μg) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-CDK2 (polyclonal) coupled to protein A beads using histone H1 as substrate. *B*, quantification of the histone H1-associated cdk2 kinase activities by scintillation counting, as described previously (16). *C*, relative percentage of cells in different phases of the cell cycle for each cell line was calculated from flow cytometric measurements of DNA content.

cells synchronized by double-thymidine block. Similar to p21, p27 levels were not induced further in G_1 of tumor cells synchronized by double-thymidine block. This suggests that the induction of p27 during lovastatin synchronization (Fig. 3*B*) is not a cell cycle-dependent effect, but an effect of lovastatin. p16 levels were minimally and constitutively detected in normal cells, but only upon prolonged exposure times (*i.e.*, 10 min *versus* 10–30 s for the other CKIs). p16 was constitutively overexpressed throughout the cell cycle in tumor cells. p15 was expressed throughout the normal cell cycle and was not detected in tumor cells even under prolonged exposure of the Western blots (*i.e.*, 24 h).

To compare the kinase activity associated with cdk2 in normal and tumor cells, we measured the phosphorylation of histone H1 in anti-cdk2 immunoprecipitates prepared from synchronous cell extracts

[(16) and Fig. 4*B*]. There was a significant difference between normal and tumor cells in the timing of cdk2 activity as described previously (16). In normal cells, the cdk2-associated kinase activity is cell cycle regulated, coinciding with the increased levels of cyclins E and A protein expression as described previously (16). Conversely, cdk2 remains catalytically active throughout the cell cycle in tumor cells, resulting in a near constitutive pattern of histone H1 phosphorylation [(16) and Fig. 4*B*]. Therefore, the observed decrease in cdk2-associated kinase activity in lovastatin-treated MDA-MB-157 cells (Fig. 3*C*) is most likely a direct result of inhibition mediated by the induction of p21 and p27 proteins by lovastatin.

The studies reported here represent a comprehensive analysis of the expression of four CKI mRNAs and proteins including both the $p21^{CIP1/WAF1}$ and Ink4 families in normal and tumor breast epithelial cells. Our data indicate that striking differences exist among the individual CKIs between normal and tumor cells. p21 mRNA and p15 protein expression are consistently higher in normal cells while p27 and p16 mRNA and protein are overexpressed in some tumor cells. Additionally, the cell cycle expression levels of the CKI proteins are different, which suggests that these inhibitors may be functionally distinct throughout the cell cycle. We have found that the drug lovastatin is capable of inducing p21 and p27 protein in a cell-specific manner via a cell cycle-independent mechanism.

Lovastatin, an inhibitor of HMG CoA reductase (the first enzyme in the isoprenyl lipid biosynthetic pathway), is a widely used drug for the treatment of hypercholesterolemia. Lovastatin prevents the first step of cholesterol synthesis, which is the conversion of HMG into mevalonic acid. The blockage of this pathway also prevents the isoprenylation of several proteins such as Ras, Rap, and G by farnesyl, a downstream product of the pathway. This inhibition of isoprenylation blocks the function of the aforementioned proteins (26, 27). Apart from its inhibitory action on HMG CoA reductase, lovastatin has also been used as an effective agent in cell synchronization for both tumor and normal cells (12, 13).

Recently, Hengst *et al.* (9, 10) reported an elevation of p27 in lovastatin-arrested HeLa cells. This increase was attributed to a cell cycle effect since a similar increase was observed in cells synchronized by density-mediated arrest and thymidine and nocodazole blocks (10). However, the increase in p27 levels, in density-mediated arrested fibroblasts, was much lower than that in lovastatin-treated HeLa cells. This was attributed to imperfect synchronization of the fibroblast cells, indicating that the increases in p27 levels seen might be due to experimental design. Furthermore, HeLa cells are derived from a cervical carcinoma and carry the human adenopapilloma virus, whereas most cancer cell lines in general do not carry this virus.

We have shown here that although lovastatin is capable of inducing both p21 and p27 in human breast tumor cells, this induction is not due to cell cycle synchronization effects of lovastatin. The induction of the inhibitors was not observed using other methods of cell synchronization such as double-thymidine block in normal and tumor cells or growth factor deprivation in normal cells. Furthermore, we used both normal and tumor cells that were derived from human mammary epithelial cells which are representative of most types of cancer, since more than 90% of all human cancers are of epithelial origin. Although lovastatin is capable of inducing these inhibitors in human epithelial cells, the mechanism of induction is not through arrest of cells in a specific phase of the cell cycle, but through a lovastatin, drug-mediated effect. Whether the CKI induction following lovastatin treatment is due to inhibition of any one reaction of the cholesterol biosynthesis pathway remains to be elucidated.

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Cell Biology

Cyclin E, a redundant cyclin in breast cancer

(cell cycle/retinoblastoma/p16/redundancy)

JULIE GRAY-BABLIN*, JUAN ZALVIDE†, M. PAT FOX*, CHRIS J. KNICKERBOCKER*, JAMES A. DECAPRIO†, AND KHANDAN KEYOMARSI*‡

*Division of Molecular Medicine, Laboratory of Diagnostic Oncology, Wadsworth Center, Albany, NY 12201; and †Division of Neoplastic Disease Mechanisms, Dana–Farber Cancer Institute, Boston, MA 02146

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ABSTRACT **Cyclin E is an important regulator of cell cycle progression that together with cyclin-dependent kinase (cdk) 2 is crucial for the G_1/S transition during the mammalian cell cycle. Previously, we showed that severe overexpression of cyclin E protein in tumor cells and tissues results in the appearance of lower molecular weight isoforms of cyclin E, which together with cdk2 can form a kinase complex active throughout the cell cycle. In this study, we report that one of the substrates of this constitutively active cyclin E/cdk2 complex is retinoblastoma susceptibility gene product (pRb) in populations of breast cancer cells and tissues that also overexpress p16. In these tumor cells and tissues, we show that the expression of p16 and pRb is not mutually exclusive. Overexpression of p16 in these cells results in sequestering of cdk4 and cdk6, rendering cyclin D1/cdk complexes inactive. However, pRb appears to be phosphorylated throughout the cell cycle following an initial lag, revealing a time course similar to phosphorylation of glutathione *S*-transferase retinoblastoma by cyclin E immunoprecipitates prepared from these synchronized cells. Hence, cyclin E kinase complexes can function redundantly and replace the loss of cyclin D-dependent kinase complexes that functionally inactivate pRb. In addition, the constitutively overexpressed cyclin E is also the predominant cyclin found in p107/E2F complexes throughout the tumor, but not the normal, cell cycle. These observations suggest that overexpression of cyclin E in tumor cells, which also overexpress p16, can bypass the cyclin D/cdk4-cdk6/p16/pRb feedback loop, providing yet another mechanism by which tumors can gain a growth advantage.**

Progression through the eukaryotic cell cycle is mediated both positively and negatively by a variety of growth regulatory proteins (1–3). Cyclins and their catalytic cyclin-dependent kinase (cdk) partners act positively to propel a cell through the proliferative cycle (4, 5). Activation of cyclin-cdk complexes results in a cascade of protein phosphorylations that ultimately induce cell cycle progression (1, 4). Although the identity of downstream substrates and effectors of cyclin-cdks remains to be firmly established, it is commonly believed that cdk-mediated phosphorylations manifest cell cycle regulation via inhibition of growth inhibitory signals and activation of proteins necessary for each stage of the cell cycle (6). A putative, well-characterized substrate for the G1 cyclins is retinoblastoma susceptibility gene product (pRb; refs. 7 and 8). This protein is sequentially phosphorylated during the cell cycle presumably through the concerted activity of different cyclin-cdk complexes (9–11). This phosphorylation is required for cell cycle progression, and the hypophosphorylated form of pRb inhibits cell cycle progression by tethering and inactivating transcription factors of the E2F family, which are required for the transactivation of S phase-specific proteins, including dihydrofolate reductase, cyclin A, and thymidylate synthase (12–14). The phosphorylation of pRb results in the release of E2F transcription factors, freeing them to stimulate transcription of growth-promoting target genes.

Inhibition of pRb phosphorylation, therefore, represents a potent form of growth inhibition. Such inhibition has recently been exemplified through the characterization of cyclin-dependent kinase inhibitor proteins (reviewed in refs. 15 and 16). To date, these proteins exist as two functionally and structurally distinct groups typified by p21 and its homologues p27 and p57, as well as p16 and p15 and their related homologues (17, 18). As potential tumor suppressors, the cyclin-dependent kinase inhibitor genes have been studied extensively to evaluate the possible contribution of cyclin-dependent kinase inhibitor-specific genomic mutations to neoplastic transformation (17). In particular, the gene encoding p16, or multitumor suppressor 1, on chromosome 9p21 has been postulated to encode a tumor suppressor and has been demonstrated to be mutated in a wide variety of tumor-derived cell lines (19–22).

A curious finding has ensued from the analysis of p16 in cancer; although both pRb and p16 are often mutated in human cancers, these mutations seem mutually exclusive (23–26). This inverse correlation has been established in various tumor cell types both *in vitro* and *in vivo*. A logical conclusion then is that these proteins, which act similarly to inhibit cell cycle progression, are differentially regulated by a common pathway, perhaps involving a negative feedback loop. In fact, the growth suppression mediated via p16 overexpression has been shown to be definitively correlated with pRb status (27, 28). Thus, p16 inhibition of cell proliferation is evident only in cells expressing wild-type pRb. As an inhibitor of the putative pRb kinases cdk4 and cdk6, p16 is thought to bind, inhibit, and sequester these cdks, thereby rendering cyclin-D orphan with respect to cdk association. Some groups have postulated that p16 expression is regulated by pRb or by a feedback mechanism involving pRb (29), and it has been demonstrated by others that p16 is transcriptionally regulated by pRb (30). Such a mechanism would permit high levels of p16 to be expressed only when pRb is inactivated, by hyperphosphorylation, genomic mutation, or association with transforming viral oncoproteins. Although not without exception, the inverse correlation of these two proteins, particularly in breast epithelial cells, may represent a tightly regulated feedback mechanism.

In this report, we have identified and characterized an exception to the pRb/p16 inverse correlation rule. In the cell line MDA-MB-157, pRb is wild-type and phosphorylated, and p16 is significantly overexpressed and effectively binds cdk4 and cdk6, thus preventing cyclin D1 from binding to these kinases. We also have demonstrated that although cyclin D1-cdk4 and cyclin D1-cdk6 complexes are inactivated by p16, pRb is progressively synthesized and phosphorylated during the cell cycle. Cyclin D1, cdk4, and cdk6 are not overexpressed in this cell line; however, cyclin E is overexpressed, and its

Abbreviations: cdk, cyclin-dependent kinase; pRb, retinoblastoma susceptibility gene product; GST-Rb, glutathione *S*-transferase retinoblastoma.

‡To whom reprint requests should be addressed at: Wadsworth Center, Empire State Plaza, P.O. Box 509, Albany, NY 12201. e-mail: keyomars@wadsworth.org.

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levels and associated kinase activity remain constitutively high during all phases of the cell cycle. In addition, cyclin E-cdk2 complex can phosphorylate glutathione *S*-transferase retinoblastoma (GST-Rb) throughout the cell cycle. We conclude, therefore, that there exists a functional redundancy among the cyclins such that overexpression of cyclin E may compensate for the inactivation of cyclin D complexes by p16 with respect to the pRb phosphorylation and cell cycle progression.

METHODS

Cells Lines, Culture Conditions, and Tissue Samples. The culture conditions for all cell lines used in this study were described previously (31, 32). Snap-frozen surgical specimens from patients diagnosed with breast cancer were obtained from the Quantitative Diagnostic Laboratories (Almhurst, IL). 76N normal mammary epithelial cell strain and MDA-MB-157 tumor cell line were synchronized at the G_1/S boundary by a modification of the double thymidine block procedure (33) as described (32). For each time interval, 10^6 cells were subjected to FACScan analysis as described (32, 34).

Western Blot and Immune Complex Kinase Analysis. Cell lysates and tissue homogenates were prepared and subjected to Western blot analysis as described (31, 35). Primary antibodies used were monoclonal antibodies to cyclins E and D1 (Santa Cruz Biochemicals), cdk4 (Transduction Laboratories, Lexington, KY), pRb (PharMingen), and p16 (J.A.D.); and polyclonal antibodies to cdk6 (Santa Cruz Biochemicals) and cyclin A (a gift from J. W. Harper, Baylor College of Medicine, Houston). Immunoprecipitations and H1 kinase assays were performed as described (32, 36). Briefly, for H1 kinase and GST-Rb kinase assays, 500 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with polyclonal antibody to cyclin E. Immunoprecipitates were then incubated with kinase buffer containing either 5 μg of histone H1 or 1 μg of purified GST-Rb, 60 μM cold ATP, and 5 μCi of [γ-^{32}P]ATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were then run on a SDS/13% PAGE gel. The gel was then stained, destained, dried, and exposed to x-ray film.

For immunoprecipitation followed by Western blot analysis, 250 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with either monoclonal antibody to p16, polyclonal antibody to cyclin D1 obtained from M. Pagano (Mitotix, Cambridge, MA) (37), or monoclonal antibody to cyclin D1-clone HD33 (a gift from E. Harlow and C. Ngwu, Massachusetts General Hospital Cancer Center, Boston) in lysis buffer as described above. The immunoprecipitates were then electrophoresed on a SDS/13% PAGE, transferred to Immobilon P, blocked, and incubated with either polyclonal antibody to cdk4 obtained from M. Pagano (Mitotix) (37) or cdk6 as described in the figure legend.

Gel Retardation Assays. Whole-cell extracts were prepared as described (31, 35), and 15 μg of protein were used per lane. Binding reactions were performed as described elsewhere (13, 38). The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human dehydrofolate reductase promoter (DHFR WT) (13). For antibody perturbation experiments, 2 μl (200 ng) of rabbit polyclonal antibody to cyclin E (Upstate Biotechnology, Lake Placid, NY) was added.

RESULTS

Overexpression of p16 and Absence of Cyclin D1/cdk4-D1/cdk6 Complexes in a Breast Cancer Cell Line with Functional Rb Protein. A panel of 13 breast cell lines was surveyed for the correlation of p16 and Rb status, as well as association of p16 and cyclin D1 with cdks 4 and 6 (Fig. 1). The cell lines used include three proliferating normal mammary epithelial cell strains obtained from reduction mammoplasties and used at early passages, one near diploid normal-immortalized breast epithelial cell line and nine tumor cell lines with different cyclin E levels, estrogen receptor, and p53 status as outlined in Table 1.

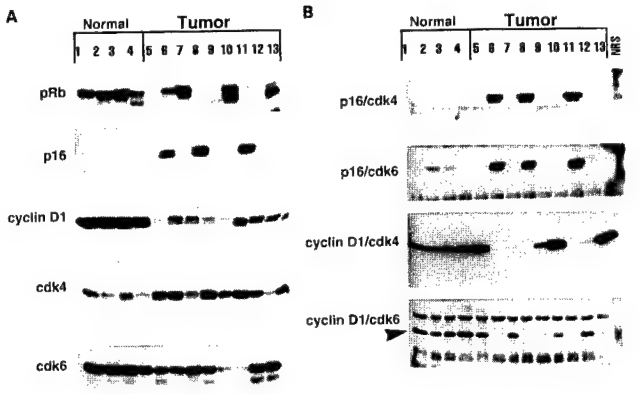

FIG. 1. Expression and complex formation of p16/pRB pathway proteins in normal and tumor-derived breast epithelial cells. (*A*) Western blot analysis: exponentially growing normal and tumor cells were subjected to Western blot analysis using 50 μg of protein for each cell line in each lane of either a 6% (pRb), 13% (cyclin D1, cdk4, and cdk6), or 15% (p16) acrylamide gel and blotted as described. The same blot was reacted with cyclin D1, cdk4, and cdk6 affinity-purified antibodies. The blots were stripped between the three antibodies in 100 mM 2-mercaptoethanol, 62.5 mM Tris·HCl (pH 6.8), and 2% SDS for 30 min at 55°C. (*B*) Immune-complex formation: for immunoprecipitation followed by Western blot analysis, equal amounts of protein (500 μg) from cell lysates prepared from each cell line were immunoprecipitated with either monoclonal antibody to p16 (p16/cdk4 and p16/cdk6), polyclonal antibody to cyclin D1 (cyclin D1/cdk4), or a monoclonal antibody to cyclin D1 (cyclin D1/cdk6), coupled to protein A/G beads, and the immunoprecipitates were washed, boiled for 3 min, separated by SDS/13% PAGE, blotted to Immobilon membranes, and hybridized with either polyclonal antibody to cdk4 (p16/cdk4), polyclonal antibody to cdk6 (p16/cdk6 and cyclin D1/cdk6; arrow pointing to the complexed protein), or monoclonal antibody to cdk4 (cyclin D1/cdk4). The list of normal and tumor cell lines is presented in Table 1 using identical numbers.

We examined the expression of pRb by direct immunoblotting with a monoclonal antibody in which the presence of functional pRb is inferred from the presence of higher molecular weight-hyperphosphorylated forms of the protein. This analysis revealed that besides three tumor cell lines (Fig. 1*A*, lanes 8, 11, and 12; i.e., MDA-MB-436, HBL-100, and Hs-578T) in which pRb is either mutated (42), inactive due to its binding to simian virus 40 large T antigen, or not expressed, pRb is present and functional in all of the other cell lines examined. Furthermore, in all of the pRB-positive cell lines, there are at least two pRb bands present representing different phosphorylation states of pRb. (Due to different levels of pRb expression in each of the cell lines, longer exposures were used to evaluate presence of slower migrating, functional form of pRb, specifically in lanes 1, 2, and 5; data not shown). Next, we correlated the expression of p16 levels with pRb status and found that p16 is overexpressed in three cell lines (Fig. 1*A*, lanes 6, 8, and 11), two of which Rb has been functionally compromised (i.e., MDA-MB-436 and HBL-100). It is curious that in MDA-MB-157, which contains a wild-type pRb, p16 is also markedly overexpressed (Fig. 1*A*, lane 6). Hence, MDA-MB-157, in which cyclin E is severely overexpressed (Table 1; refs. 31, 32), is one exception to the reciprocal p16/Rb correlation rule.

Because overexpression of cdk4, cdk6, or cyclin D1 could counteract the inhibitory effect caused by the overabundance of p16, we also measured the relative levels of these proteins in all 13 cell lines (Fig. 1*A*). Western blot analysis with cyclin D1, cdk4, and cdk6 revealed that these proteins were not overexpressed in MDA-MB-157 cell line relative to the other 12 cell lines examined, suggesting that the overexpressed p16 may adequately sequester cdk4 and cdk6 away from cyclin D1, rendering it inactive. To test this hypothesis, we performed a series of two-step immunoprecipitations followed by Western blot analysis (Fig. 1*B*). When p16 immunoprecipitates were separated on denaturing gels, transferred to poly(vinylidene

Table 1. Characterization of normal and tumor-derived breast epithelial cells

Cell lines	Cell types	Estrogen receptor (31)	p53	Cyclin E (31, 32)	pRb*
1. 70N	N-mortal	−	+ (39)	+	+
2. 81N	N-mortal	−	+ (39)	+	+
3. 76N	N-mortal	−	+ (39)	+	+
4. MCF-10-A	N-immortalized	−	+ (40)	+	+
5. MCF-7	A (pe)	+	+ (40)	+++	+
6. MDA-MB-157	C (pe)	−	− (40)	++++++	+
7. MDA-MB-231	A (pe)	−	− (40)	++++	+
8. MDA-MB-436	A	−	− (41)	+++++	−
9. T47D	DC (pe)	+	− (40)	++	+
10. BT20	C	+	+ (40)	++	+
11. HBL-100	T (bm) SV40 transformed	−	− (40)	+++	−
12. HS-578T	DC	−	− (40)	++++	−
13. ZR75T	IDC	+	+ (40)	+++	+

Cell type, estrogen receptor (ER), p53, and cyclin E status as determined in indicated references. +, wild type; ++(++++), varying degrees of overexpression with MDA-MB-157 showing the highest degree (64-fold, hence 6 + s) of cyclin E overexpression. N, normal breast cells from reduction mammoplasty; A, adenocarcinoma; pe, pleural effusion; C, carcinoma; DC, ductal carcinoma; T(bm), tumor breast milk; SV40, simian virus 40; IDC, infiltrating DC; −, mutant or not expressed.
*pRb status is adopted from Fig. 1, in which + indicates wild type and present in hypo- and hyperphosphorylated forms, and − indicates mutated or virally bound and inactive.

difluoride) membrane, and blotted with antiserum to cdk4 or cdk6, p16 was capable of forming a complex with both cdk4 and cdk6 in the three tumor cell lines in which p16 is overexpressed. Curiously, p16 was also capable of forming a complex with cdk6 in normal breast cell strains in which no overexpression of p16 or cdk6 was noted. However, cyclin D1 immunoprecipitates that were separated and blotted with antibodies to cdk4 or cdk6 revealed that, in the normal cell strains, cyclin D1 formed a complex with cdk4 and cdk6, suggesting that p16 did not completely sequester these kinases from cyclin D1. On the other hand, in tumor cells in which p16 is overexpressed, no complexes were formed between cyclin D1 and cdk4 or cdk6, suggesting that in these three tumor cell lines enough p16 is overexpressed to sufficiently sequester cdk4 and cdk6 away from cyclin D1, preventing it from forming complexes with these kinases (Fig. 1*B*). Collectively, these data provide evidence for the absence of cyclin D1/cdk complexes in a breast cancer cell line with a functional Rb protein.

Cyclin E-Associated Kinase Phosphorylates pRb in the Absence of Cyclin D1/cdk4 or Cyclin D1/cdk6 Complexes in Tumor Cells. To examine the cell cycle regulation of pRb in normal and tumor cells, we synchronized both cell lines by double thymidine block and analyzed the pattern of pRb expression and phosphorylation by Western blot analysis (Fig. 2*A*). Synchrony of both cell types at several times after release from the block was monitored by flow cytometry (Fig. 2*C*). At various times after release from treatment for synchronization, cells were harvested, and extracted proteins were analyzed on Western blots with antibodies to pRb and cyclins E and A (Fig. 2*A*). In normal 76N cells, the pattern of synthesis and phosphorylation of pRb, as well as expression of cyclin E and cyclin A proteins, is consistent with that seen for other normal cell types, with levels rising before S phase and oscillating thereafter in the cell cycle (8, 43, 44). In addition, pRb is present mainly in the hyperphosphorylated form at G_1/S boundary up to G2, where the levels drop to resume again at G_1. Furthermore, there is only one major form (i.e., 50 kDa) of cyclin E protein detected. However, in the tumor cells, pRb and cyclin E proteins do not appear to be cell cycle-regulated. pRb is induced and phosphorylated shortly after release from thymidine block and remains in that phosphorylated state throughout the cell cycle. In addition, multiple isoforms of cyclin E protein are present with similar signal intensities and banding patterns during the time intervals examined. In the same tumor cell extracts, cyclin A protein is cell cycle-regulated with peak levels coinciding with peak S and early M phase. Hence, it appears that in this tumor cell line, pRb and cyclin E are abnormally regulated during the cell cycle.

To decipher whether cyclin E-associated kinase is responsible for the phosphorylation of pRb, cells were immunoprecipitated with cyclin E antibody and used in kinase assays with either histone H1 or a recombinant GST-Rb fusion protein as substrates (Fig. 2*B*). In normal cells, cyclin E-associated kinase is capable of phosphorylating histone H1 and is cell cycle-regulated, coinciding with the levels of cyclin E protein expression (Fig. 2*A*). However, the same cyclin E immunoprecipitates prepared from normal cells were not capable of phosphorylating GST-Rb (Fig. 2*B*). In tumor cells, on the other hand, cyclin E is not cell cycle-regulated and remains in a catalytically active complex throughout the cell cycle, resulting in a constitutive pattern of histone H1 and GST-Rb phosphorylation. Finally, the timing of pRb expression in the tumor cell cycle (Fig. 2*A*) is similar to the timing of phosphorylation of GST-Rb by cyclin E immunoprecipitates (Fig. 2*B*). These observations suggest that overexpression of cyclin E results in an active kinase complex throughout the cell cycle capable of phosphorylating not only histone H1, but also GST-Rb. Hence, in tumor cells that overexpress p16, resulting in the inactivation of cyclin D1/cdk4 or cyclin D1/cdk6 complexes, pRb can still get phosphorylated by cyclin E-associated kinase.

Overexpression of Cyclin E and p16 in Breast Tumor Tissues Is Correlated with Functional pRb. Because the lack of inverse association of pRb and p16 was observed in only one of three breast tumor cell lines overexpressing p16 (Fig. 1*A*), we were interested in deciphering the frequency at which such a phenomenon would occur in breast tissue samples. Therefore, we examined 20 tumor tissue specimens obtained from breast cancer patients. Table 2 lists estrogen and progesterone status, ploidy, and proliferation index expression as measured by immunofluorescence with the respective antibodies followed by image analysis as described (45, 46). We also analyzed the expression of cyclin E, p16, and pRb in these samples by Western blot analysis. The results revealed that cyclin E was severely overexpressed and present in lower molecular weight forms in 18 of 20 tissue samples, which is consistent with the role of cyclin E as a prognosticator for breast cancer (31, 35, 48). The pattern of cyclin E expression observed in these tumor specimens was similar to those used in a previous

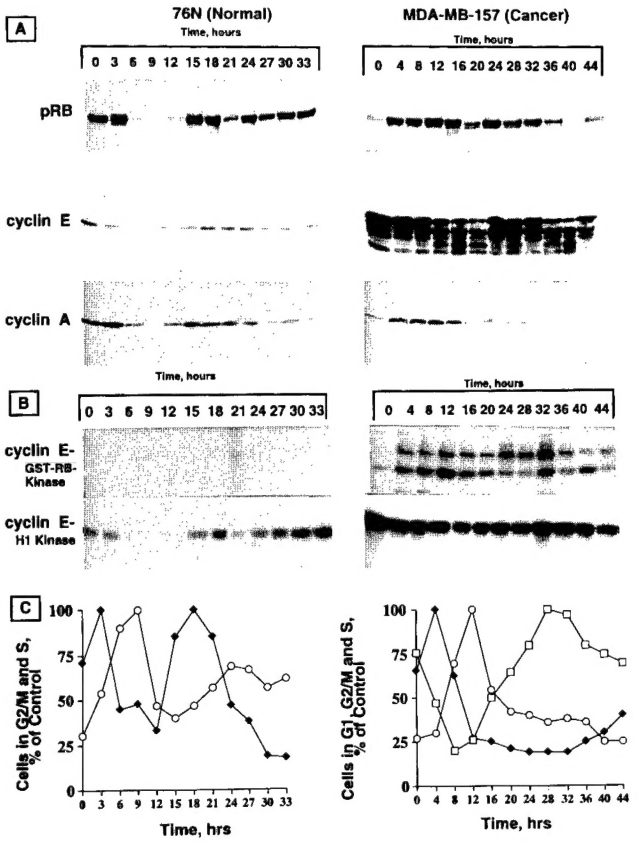

FIG. 2. Phosphorylation of pRb in synchronized population of tumor versus normal cells. Both cell types were synchronized by double thymidine block procedure. At the indicated times after release from double thymidine block, cell lysates were prepared and subjected to Western blot analysis (*A*) and histone H1 or GST-Rb kinase analysis (*B*). Protein (50 μg) for each time point was applied to each lane of either a 6% (pRb) or 10% (cyclins E and A) acrylamide gel and blotted as described. The same blot was reacted with cyclin E monoclonal (HE12) and cyclin A affinity-purified polyclonal antibodies. The blots were stripped between the two assays as described for Fig. 1. For kinase activity, equal amounts of proteins (600 μg) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-cyclin E (polyclonal) coupled to protein A beads using either histone H1 or purified GST-Rb as substrates. (*C*) The relative percentage of cells in different phases of the cell cycle for each cell line at various times after release from double thymidine block was calculated from flow cytometric measurements of DNA content. ◆, cells in S phase; ○, cells in G_2/M phase; □, cells in G_1 phase.

study (49) showing presence of lower molecular weight forms of cyclin E with increasing stage of the disease. It is interesting that most of the tumor specimens that showed an overexpression of cyclin E also were negative for estrogen and progesterone receptors. A negative steroid receptor status is indicative of poor response to endocrine and cytotoxic chemotherapy characteristics of very aggressive breast tumors (50). Furthermore, p16 was overexpressed in 7 (i.e., KK-005, 086, 147, 173, 190, 369, and 399) of the 20 samples examined. Three of these seven samples had a defect in pRb expression, whereas in the remaining four samples (i.e., KK-005, 147, 173, and 369), pRb was expressed and present in multiple bands, suggesting a functional protein. In addition, cyclin E was severely overexpressed in all four p16/pRb double-positive samples. Hence, these observations suggest that *in vivo*, in breast cancer tissues that overexpress cyclin E, overexpression of p16 is not always accompanied by a defect in pRb, consistent with results obtained with MDA-MB-157 cell line. Cyclin E, which is overexpressed and present in lower molecular weight forms in these tumor tissue samples, may be capable of phosphorylating pRb in the absence of functional cyclin D-containing complexes *in vivo* as well as in cell lines.

Cyclin E Is Present in E2F Complexes Throughout the Cell Cycle of Tumor, but Not Normal, Cells. One of the major targets of growth regulation by pRb is the E2F family of transcription factors. During the G1 phase of the cell cycle, underphosphorylated pRB binds to E2F and represses its transcriptional activity. Phosphorylation of pRb by cyclins during late G_1 and S phase release E2F, which in turn leads to activation of the transcription of genes important for cell cycle progression. Similarly, p107 and p130, two pRb-related proteins, regulate the transcriptional activity of E2F. In addition, both cyclins A and E can bind to p107 and p130 while in complex with E2F. Although the significance of this association is not known, it has been suggested that it regulates the transcriptional activity of E2F.

To determine whether the cyclin E overexpression in the tumor cell lines affected the E2F DNA binding complexes throughout the cell cycle, we performed bandshift assays using an oligonucleotide with an E2F binding site as a probe (Fig. 3). As a control, extracts from a synchronized population of normal cells were prepared. As described (13), normal cells contained several E2F complexes that were present at various times in the cell cycle. The disappearance of E2F complexes at 6, 9, and 12 h after release from the thymidine block occurred when the cells were enriched for G_2/M (Fig. 3*A*; ref. 13). The complex marked with an arrow contained the pRB-related protein p107 and cyclin A, as determined by antibody supershift analysis (data not shown). Addition of cyclin E antibody did not have any effect on the mobility of this complex (Fig. 3*A*), suggesting that cyclin E is not the predominant cyclin in the p107/E2F complex in normal cells. On the other hand, in extracts prepared from tumor cells, E2F complexes were present throughout the cell cycle, and no loss of these complexes was observed during G_2/M. The complex marked with an arrow could be disturbed with anti-p107 and partially with anti-cyclin A antibodies (data not shown). The addition of an anti-cyclin E antibody resulted in a supershift of a large proportion of the complex, suggesting that most of the p107-E2F complex contained cyclin E (Fig. 3*B*). Addition of antibodies to cyclin A and cyclin E to the same extract did not result in the appearance of any different complexes than when both antibodies were added independently (data not shown), suggesting that both cyclins did not form part of the same complex. The association of cyclin E with the E2F complexes in tumor cells paralleled the constitutive expression of cyclin E throughout the cell cycle (Fig. 2*A*, *Right*). Hence, overexpression of cyclin E in tumor cells was capable of forming a major complex with p107 and E2F. This is a second example of how overexpression and constitutive expression of cyclin E could result in a dual role for this cyclin allowing redundancy in function.

DISCUSSION

The interplay between cyclin D1/cdk4-cdk6/p16/pRb has been implicated as a crucial G_1 phase-controlling pathway that becomes frequently deregulated in many types of cancer. Any mutations giving rise to an imbalance in any one of these proteins may therefore result in a cell growth advantage leading to tumorigenesis. In this model, overexpression of p16 would prevent cdk4/cdk6 from phosphorylating pRb, and lead to a G_1 block (27–29). Thus, p16 is thought to negatively regulate the cell cycle (51). In fact, several studies have documented that primary tumors that showed expression of functional pRb protein did not express p16 protein (due to mutations in the gene) and, conversely, cells that expressed p16 protein did not have a detectable pRb protein (23–26). These studies suggest a link between D-type cyclins, cdk4/cdk6, pRb, and p16, such that overexpression of cyclin D1, inactivation of pRb, or loss of p16 may have equivalent consequences for loss of normal growth control. In addition, this model predicts a lack of functional redundancy of this pathway with other cell cycle regulatory proteins.

Even though many studies have corroborated the p16/pRb inverse correlation model, there also has been documentation to the contrary. For example, in their analysis of pRb and p16 expression in lung cancers, Otterson *et al.* (25) reported that 14%

Table 2. Correlation of p16 and pRb status in a series of breast carcinomas

Patient ID no.	ER/PR*	DNA index/ploidy*	Proliferation index (%)*	Cyclin E†	p16†	pRb†
KK005	−/−	1.18/Aneuploid	12.2 (H)	+++	++++	+
KK017	−/−	1.72/Aneuploid	1.5 (L)	++++++	±	+
KK020	−/−	1.73/Aneuploid	14.1 (H)	+++++	−	−
KK036	+/−	1.84/Tetraploid	3.3 (L)	++	±	+
KK061	−/−	ND	ND	++++	±	−
KK070	+/+	ND	ND	+	±	−
KK076	−/−	2.08/Tetraploid	12.5 (H)	+++	±	−
KK086	−/−	1.50/Aneuploid	36.0 (H)	+++++	++	−
KK147	ND	ND	ND	+++++	+++	+
KK173	+/−	1.91/Tetraploid	30.2 (H)	+++++++	++++	+
KK190	−/−	2.09/Tetraploid	31.8 (H)	+++++++	+++	−
KK322	+/−	2.70/Aneuploid	30.0 (H)	+++	−	+
KK369	ND	ND	40.0 (H)	+++++++	++++	+
KK399	−/−	ND	ND	++++	++++	−
KK400	+/−	ND	ND	++++	±	−
KK407	−/−	1.89/Tetraploid	18.0 (H)	++++	−	−
KK428	−/−	1.75/Aneuploid	27.0 (H)	++++	−	−
KK429	−/−	1.71/Aneuploid	28.0 (H)	+++++	−	−
KK457	ND	ND	ND	++++++	−	+
KK458	−/−	1.96/Tetraploid	11.3 (H)	+	−	+

*Quantitation of immunohistochemical staining by image analysis was performed on sections stained with either the monoclonal antibody to estrogen receptor H222 (ER-ICA kit, Abbott), monoclonal antibody to progesterone receptor mPRI (Cell Analysis Systems, Lombard, IL), or monoclonal antibody to Ki67 (Dako) as described (45, 46). Ki67 staining determined growth fraction of the tumor. Values indicate percentage of positive staining: 1.0–7.0% is indicative of low (L) proliferation index, 7.1–11.9 is indicative of moderate (M) proliferation index, and >12.0% is indicative of high (H) proliferation index. For each case, the DNA ploidy was determined by quantitation of the DNA Feulgen stain by computerized microdensitometry as described (47). ND, not determined.

†Cyclin E, p16, and pRb levels were measured using Western blot analysis with HE12 monoclonal antibody to cyclin E (Santa Cruz Biotechnology) as described (31, 32), monoclonal antibodies to p16, and pRb as described in text. Levels of cyclin E in tumor tissue samples were correlated with 76N normal (+) and MDA-MB-157 (++++++) tumor cell lines. For example, cyclin E in MDA-MB-157 cell line is 64-fold (i.e., ++++++) overexpressed compared with 76N cell line (i.e., +) (31). Any tumor tissue overexpressing cyclin E more than MDA-MB-157 received seven +s (i.e., +++++++). p16 levels also were correlated with MDA-MB-157 (++++) cell line. Equal protein loading was monitored by reprobing blots with actin, and all blots were analyzed by densitometry using AGFA scanner and IP Lab Gel software.

of small cell lung cancers and 15% of non-small cell lung cancers examined were p16 and pRb double positives, and Sakaguchi *et al.* (52) reported that 16.4% of non-small cell lung cancers studied immunohistochemically also stained positively for both p16 and Rb protein. In addition, Gerardts *et al.* (53) report that in 43% of all carcinomas examined (breast: 5 of 20; bladder: 7 of 19; colon: 16 of 19; lung: 4 of 17), both pRb and p16 could be detected, suggesting that in common human malignancies, p16 and pRb expression is not mutually exclusive. Furthermore, Musgrove *et al.* (54) report that in 50% of breast cancer cell lines examined, $INK4^{p16}$ mRNA was expressed in the absence of any pRb mutations. Finally, Ueki *et al.* (49) show that 13% of glioblastoma cell lines examined showed neither p16 nor RB alterations, and Wang *et al.* (55) report that regardless of the status of p16 protein, all 15 melanoma cell lines examined showed the presence of pRb protein, ruling out an inverse correlation between the expression of p16 and pRb in these particular cell lines.

One possible explanation for the lack of inverse correlation between p16 and pRb may be due to overexpression of cyclin E, which could act redundantly and replace cyclin D/cdk complexes for phosphorylating pRb. In accordance with this redundancy hypothesis, Hinds *et al.* (56) first demonstrated that overexpression of several different cyclins, including cyclin E, could override the growth arrest properties of pRb in SaOS-2 cells. In addition, we had reported previously that cyclin E is severely overexpressed in all breast cancer cell lines examined (31), and overexpression of cyclin E is accompanied by its constitutive expression and activity throughout the tumor cell cycle (32). Because cyclin E is overexpressed and forms a complex with cdk2 constitutively, the active complex can act upstream of pRb and phosphorylate it even when cyclin D is inactive due to overexpression of p16. To test this model, in this study we used a breast cancer cell line that exemplified an exception to the inverse correlation rule of p16/pRb. In this tumor cell line (MDA-MB-157), cyclin E is markedly overexpressed and present in lower molecular weight isoforms, p16 is also overexpressed, and pRb is not mutated and detectable in both its hypo- and hyperphosphorylated forms. Under these conditions, we show that p16 binds to both cdk4 and cdk6 and inhibits the binding of cyclin D1 to these cdks. We also provide evidence that, in synchronized populations of MDA-MB-157 cells, pRb is phosphorylated throughout the cell cycle following an initial lag, revealing a time course similar to phosphorylation of GST-Rb by cyclin E immunoprecipitates prepared from these synchronized cells. This analysis suggests that cyclin E/cdk2, and not cyclin D/cdk4-cdk6, is a candidate kinase complex capable of phosphorylating pRb throughout the cell cycle of this tumor cell line.

To directly examine the lack of inverse correlation of p16 and pRb *in vivo*, we document in Table 2 that in breast tumor specimen obtained from breast cancer patients in whom cyclin E is markedly overexpressed and p16 also is overexpressed, pRb is detectable in both its hypo- and hyperphosphorylated forms. These studies suggest that phosphorylation of pRb under conditions in which cyclin D/cdk complexes are rendered inactive is not an artifact of the culture conditions and occurs *in vivo*.

Because cyclin E is constitutively expressed in MDA-MB-157 cancer cells and is present during times in the cell cycle when cyclin A is not detected (see Fig. 2), it followed that cyclin E could also replace cyclin A-containing complexes. In fact, as displayed in Fig. 3, cyclin E can function redundantly and replace cyclin A in E2F complexes with cdk2 and p107 in tumor cells. In normal cells, cyclin E was not detected in complex with the pRb-related proteins p107 and p130 and with E2F during the late G_1 and early S phase of the cell cycle. We have found that while this cyclin was a minor component of E2F DNA binding complexes in normal cells, it was a major component of this complex in MDA-MB-157 cells. It is interesting that although normal cells display a down-regulation of E2F DNA binding activity in the G_2/M phases of the cell cycle, MDA-

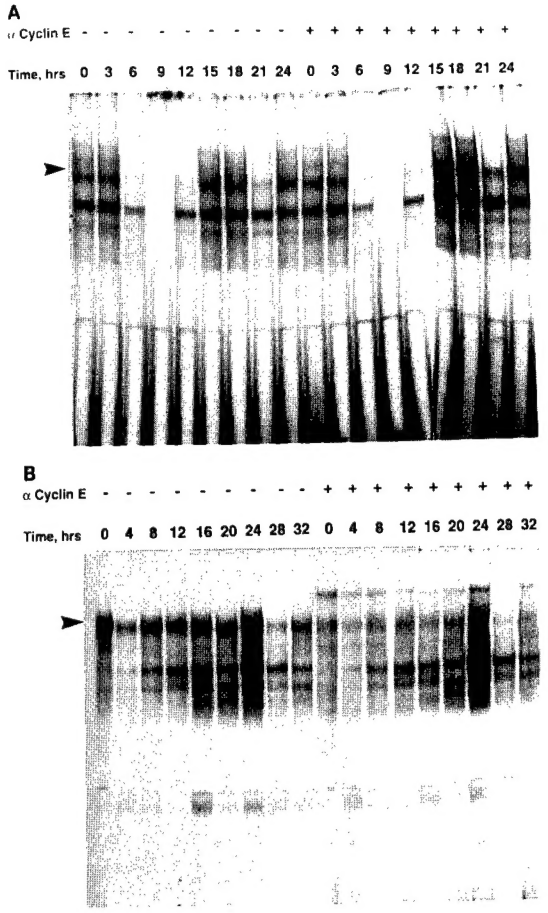

FIG. 3. Cyclin E is the predominant cyclin in p107/E2F complexes in tumor cells. E2F complexes were analyzed by gel retardation assays using cell lysates (15 μg) prepared from synchronized populations (see Fig. 2) of normal 76N cells (*A*) and tumor MDA-MB-157 cells (*B*). The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human dehydrofolate reductase promoter. The anti-cyclin E antibody (200 ng) was used to disrupt the E2F complexes.

MB-157 cells show constitutive E2F DNA binding complexes through the cell cycle. This raises the possibility that overexpression of cyclin E perturbed the regulation of E2F activity not only by promoting the hyperphosphorylation of pRb but also by perturbing the cell cycle regulation of E2F by p107.

Based on our observations in breast cancer cell lines and tumor tissue samples, we suggest an alternative order of events along the G_1 phase-controlling pathway culminating in phosphorylation of pRb. In this pathway, cyclin E would act upstream of pRb bypassing cyclin D/cdk4 and giving the tumor cells a selective growth advantage even in the presence of high levels of p16. Hence, abrogation of cyclin D1, cdk4/cdk6, or p16 will not have any effect on the phosphorylation of pRb, which will be accomplished by cyclin E/cdk2 in these cells leading to a deregulated progression through G_1. Our data also demonstrate that cyclin D1 is not required for G_1 progression in tumor cells that exhibit an overexpressed cyclin E and a wild-type pRb. As a result, the function of cyclin D1 is dispensable not only in cell lines in which pRb is inactivated as described (57), but also in cell lines in which cyclin E is overexpressed and constitutively active (ref. 58 and this study). Finally, this study provides evidence for a lack of functional link between p16 and pRb, suggesting that in subpopulations of breast cancers, pRb is not a major substrate for the inhibitory activity of the p16 product. Hence, certain populations of tumor cells can overcome the role of p16 as a tumor suppressor protein by providing a redundant pathway to inactivate pRb and provide a growth advantage to the cells.

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